Division of Pharmaceutical Biosciences
Faculty of Pharmacy
University of Helsinki
Finland

Specific Cell – Biomaterial Interactions for *In Vivo*-Like *In Vitro* Tissue Models from Human Pluripotent Stem Cells

Riina Harjumäki

ACADEMIC DISSERTATION

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Supervisors
Associate Professor Monika Österberg
Division of Bioproducts and Biosystems
School of Chemical Engineering
Aalto University
Finland

Adjunct Professor Yan-Ru Lou
Division of Pharmaceutical Biosciences
Faculty of Pharmacy
University of Helsinki
Finland

Ph.D. Juan Jose Valle-Delgado
Division of Bioproducts and Biosystems
School of Chemical Engineering
Aalto University
Finland

Professor Marjo Yliperttula
Division of Pharmaceutical Biosciences
Faculty of Pharmacy
University of Helsinki
Finland

Members of steering group
Professor Timo Otonkoski
Research Programs Unit, Molecular Neurology and Children's Hospital
University of Helsinki
Finland

Professor Timo Ylikomi
Finnish Centre for Alternative Methods
School of Medicine
University of Tampere
Finland

Professor Orlando Rojas
Division of Bioproducts and Biosystems
School of Chemical Engineering
Aalto University
Finland

Pre-examiners
Associate Professor Emily Cranston
Wood Science and Chemical & Biological Engineering
University of British Columbia
Canada

Associate Professor Clemens Franz
WPI Nano Life Science Institute
Kanazawa University
Japan

Opponent
Professor Cecilia Sahlgren
Åbo Academy
Finland

Custos
Professor Marjo Yliperttula

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Abstract

There is an urgent need for better *in vitro* cell models to increase efficacy and cost-efficiency in drug development. Current simple models poorly mimic the natural *in vivo* cell environment. Human pluripotent stem cells (hPSCs) could serve as a limitless source for all the cells in the human body, but for most cell types, such as hepatocytes, efficient differentiation protocols do not exist. The signals that control cell behavior *in vivo* and *in vitro* are generated from growth factors (GFs), cell-extracellular matrix (ECM), and cell-cell interactions. The role of the ECM in cell behavior has only recently gained attention. Natural ECM of cells is a tissue-specific and complex three-dimensional (3D) array of various macromolecules. It provides physical, mechanical, and biochemical signals to cells. Mimicking the entire natural environment for cells is difficult, and it is, therefore, important to recognize the key components providing the essential signals. New materials, such as unmodified cellulose nanofibril (CNF) hydrogel, have been developed to tackle the technical difficulties that the ECM proteins have in 3D cell culture models, but the interactions of these materials with cells are not well known. Integrins with 18 subtypes are the main mediators of the cell – biomaterial interactions. The presentation and activation of these subtypes are important mediators in hPSC maintenance and differentiation. The activation of integrins can be caused by inside-out signaling through other integrins or receptors and outside-in activation through ECM molecules, divalent cations, or GFs. Hence it is vital to be able to measure these interactions in order to design good *in vitro* cell models. One of the most versatile instruments to quantify cell – biomaterial interactions and integrin activation is the atomic force microscope (AFM).

The aim of this thesis is to study the hPSC interactions with biomaterials and use this information to better understand the cell behavior *in vitro*. The adhesion data of the AFM-based colloidal probe microscopy (CPM) correlate and predict cell adhesion on materials *in vitro*. Using CPM, we quantitatively tested the role of integrin density as well as integrin activation, enabled by cell viability and divalent cations, in these interactions. We observed that ECM proteins laminin-521 and laminin-511—detected in acellular matrix produced by hepatic progenitor cells—improved hPSC differentiation to hepatic cells. Cells in 3D cultures have more *in vivo*-like functions, and we, therefore, tested if the created differentiation protocol could be used to stepwise induce hPSCs specification to hepatic organoids in a CNF hydrogel. With CPM we found that CNF has only weak, nonspecific interactions with cells and maybe therefore CNF is not providing the signals needed for hPSC differentiation. The differentiation efficiency of hPSCs in CNF hydrogel is lower compared to matrix-free suspension culture. In conclusion, this thesis provides new quantitative information about cell – biomaterial interactions with a particular focus on hPSC cells, and laminin and CNF biomaterials. The implications of these interactions on *in vitro* cell cultures and stem cell differentiation to hepatic cells are analyzed.

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Contents

List of original publications

I **Harjumäki R**, Nugroho RWN, Zhang X, Lou Y-R, Yliperttula M, Valle-Delgado JJ, Österberg M. Quantified forces between HepG2 hepatocarcinoma and WA07 pluripotent stem cells with natural biomaterials correlate with *in vitro* cell behavior. Scientific Reports, 9:7354, 2019.

II **Harjumäki R**, Zhang X, Nugroho RWN, Muhammad F, Lou Y-R, Yliperttula M, Valle-Delgado JJ, Österberg M. AFM force measurements reveal the role of integrins and their activation in cell – biomaterial interactions. Submitted manuscript.

III Kanninen LK, **Harjumäki R**, Peltoniemi P, Bogacheva MS, Salmi T, Porola P, Niklander J, Smutný T, Urtti A, Yliperttula ML, Lou YR. Laminin-511 and laminin-521-based matrices for efficient hepatic specification of human pluripotent stem cells. Biomaterials 103: 86-100, 2016.

IV Stepwise human pluripotent stem cell differentiation to hepatic organoids in three-dimensional cell cultures. Unpublished data.

The published and unpublished data are referred to in the text by their Roman numerals.

Author's contribution

Publication I

The author designed the experiments together with Prof. Monika Österberg and Ph.D. Juan Jose Valle-Delgado with the help of Ph.D. Robertus Nugroho. Prof. Marjo Yliperttula, and Ph.D. Yan-Ru Lou. The biomaterials were prepared by the author and M.Sc. Xue Zhang and biomaterial coatings by the author, Ph.D. Nugroho, Ph.D. Valle-Delgado, and M.Sc. Zhang. The cell culture and viability analysis were carried out by the author. The AFM measurements and data analysis were performed by the author, Ph.D. Nugroho, and Ph.D. Valle-Delgado. The paper was written by the author, Ph.D. Valle-Delgado and Prof. Österberg. The author had the main responsibility for the manuscript concerning the experiments, data analysis, and writing.

Publication II

The author designed the experiments together with Prof. Monika Österberg and Ph.D. Juan Jose Valle-Delgado. All the cell cultures were performed by the author. The biomaterials and biomaterial coatings were prepared, and AFM measurements performed by the author, M.Sc. Zhang, Ph.D. Nugroho, and Ph.D. Valle-Delgado. The data analysis was carried out by the author together with M.Sc. Zhang and Ph.D. Valle-Delgado. FESEM imaging and analysis were performed by M.Sc. Farooq Muhammad and SEM imaging and analysis by the group of Prof. Jeff Brinker. Silica coating was prepared by Dr. Lou. The author wrote the paper with the help of Ph.D. Valle-Delgado and Prof. Österberg. The author had the main responsibility for the manuscript concerning the experiments, data analysis, and writing.

Publication III

The experiments were designed by M.Sc. (later Ph.D.) Liisa Kanninen and Ph.D. Lou. The author helped to perform WA07 and iPS(IMR90)-4 cell culturing together with M.Sc. Kanninen, undergraduate Johanna Niklander and Ph.D. Lou. The analysis of these cells by confocal microscope, ELISA and qPCR was performed by the author, M.Sc. Kanninen, and Ph.D. Lou. Other analysis of these cells was performed by M.Sc. Kanninen, and Ph.D. Lou. The cell culturing and analysis of H9-GFP cell line were carried out by undergraduate (later M.Sc.) Pasi Peltoniemi and Ph.D. Lou. The characterization of the HepaRG-ACM by conventional PCR was undertaken by M.Sc. (later Ph.D.) Pauliina Porola and immunostaining by M.Sc. Kanninen, Mr. Peltoniemi and Ph.D. Lou. The author commented on the paper written by M.Sc. Kanninen and Ph.D. Lou.

Other unpublished data IV

The experiments were designed by the author together with Ph.D. Lou. WA07 and iPS(IMR90)-4 cell culturing was performed by the author with the help of Ph.D. Lou, undergraduate (later M.Sc.) Ara Taalas and M.Sc. Mariia Bogacheva. The live/dead analysis and qPCR were performed by the author, and the immunofluorescence was carried out together with Mr. Taalas. The author has the main responsibility for the experiments and data analysis.

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ACM	Acellular matrix
ActA	Activin A
AFM	Atomic force microscopy
AFP	Alpha-fetoprotein
ALB	Albumin
BMP	Bone morphogenetic protein
CK	Cytokeratin
CNF	Cellulose nanofibrils
Col	Collagen
COL	Collagenous triple-helical domain
CPM	Colloidal probe microscopy
DE	Definitive endoderm
DEX	Dexamethasone
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FESEM	Field emission scanning electron microscopy
FGF	Fibroblast growth factor
FN	Fibronectin
GAG	Glycosoaminoglycan
GF	Growth factor
GFR	Growth factor receptor
HBD	Heparin-binding domain
HGF	Hepatocyte growth factor
hESC	Human embryonic stem cell
hiPSC	Human induced pluripotent stem cell
hPSC	Human pluripotent stem cell
ihPSF	Immortalized human placental stromal fibroblast
IF	Immunofluorescence
LG	Laminin globular subdomain
LN	Laminin
MEF	Mouse embryonic fibroblast
MSC	Mesenchymal stem cell
NaBut	Sodium butyrate
NFC	Nanofibrillar cellulose
NME	New molecular entity
OSM	Oncostatin M

qPCR	Quantitative polymerase chain reaction
RGD	Arg-Gly-Asp
RPLP0	Ribosomal protein large P0
RT-PCR	Real time polymerase chain reaction
SCFS	Single cell force spectroscopy
SEM	Scanning electron microscopy
TGF-β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
Wnt	Wingless type

1. Introduction

The capitalized costs to develop a new molecular entity (NME), a novel drug, into markets have increased over nine-fold within 40 years, and the number of compounds in development has increased by 62% within the last decade (Paul et al. 2010; Morgan 2011; Hay 2014). Despite these efforts, productivity in research and development has decreased dramatically (Shimura et al. 2014). Some studies have suggested that development risk has remained relatively stable but that clinical trials have become more complex, and, thus, more expensive (DiMasi et al. 2003; Getz et al. 2008). Less than 10% of the drugs that enter the clinical phase are eventually going to gain market approval (Hay et al. 2014). Additionally, withdrawals from markets continue to occur. In the EU, 19 drugs were withdrawn between 2002 and 2011. The second leading cause for withdrawal was unacceptable toxicity (McNaughton 2014).

Since the costs of clinical trials are approximately two-thirds of the total NME development costs, the need for more predictive *in vitro* models to increase future clinical success is crucial (Morgan et al. 2011). The fast development of new improved, faster and cost-efficient high throughput screening and *in silico* models has not improved drug development productivity (Scannell et al. 2012). One explanation might be that the shift from animal testing to *in silico* models does not give the whole picture on complex off-target effects. Because of the ethical questions of animal models, and the fact that they are poor models due to the considerable differences in reactions to drugs between animal species, more attention needs to be paid to cell culture models (Burkina et al. 2017; Williams 2018). Predictive toxicology models have been an area showing little improvement over the past two decades (Astashkina and Graiger 2014).

Numerous cell culture systems, reagents, devices, and analysis methods have been established since the idea of culturing cells *in vitro* (Harrison et al. 1907). Despite this development, cell culture is routinely performed with simple techniques and cell types, which vary considerably from the actual situation *in vivo*. Tissue engineering aims to provide signals to cells that promote controlled cell behavior. These signals are generated from growth factors (GFs), cell – extracellular matrix (ECM), and cell – cell interactions, as well as from physical, biochemical, and mechanical stimuli (Rosso et al. 2004).

In drug development, conventionally used cell lines, such as human carcinoma and primary cell lines, have compromised functions. Carcinoma cells and immortalized cells have abnormal functions giving potentially false results. Human primary cells are expensive, difficult to obtain and have significant batch-to-batch variability. They also lose their functions fast *in vitro*. The generation of human pluripotent stem cells (hPSCs), human embryonic stem cells (hESCs) and human induced pluripotent stem

cells (hiPSCs) has revealed a new, potentially unlimited source for all cell types of the human body with normal functions (Thomson 1998; Takahashi 2007; Yu 2007). Human iPSCs derived from patients could also be used as disease models in drug testing (Williams 2018). Unfortunately, obtaining fully mature cells through differentiation has proven to be challenging and has not been successful for most of the cell types, such as hepatocytes.

The extracellular environment also affects cell functions and, thus, has recently gained attention as a potential guide for improved *in vitro* cell culture models. ECM is formed from a complex three-dimensional (3D) array of large molecules, such as glycoproteins, collagens, glycosaminoglycans, and proteoglycans, which are secreted and degraded dynamically by cells. ECM provides the physical, chemical, and biological signaling for the cells, and is critical in cell behavior and phenotype (Hynes 2009). The main mediators of this bidirectional crosstalk between cells and ECM are cell-surface receptors called integrins. Various tissues and cell types have a unique composition of ECM and integrin cassette. Despite these facts, tissue models are usually built by using general cell culture materials such as Matrigel.® The role of physical, chemical, and biological tissue specificity in ECM and the signals they provide, should be further studied and considered when planning functional *in vivo*-like *in vitro* tissue models. To date, there are only a few suitable methods to study cell – ECM interactions in more detail and quantitatively. Atomic force microscopy (AFM) has been shown to have excellent features for these interaction studies. Nevertheless, little information from these studies has yet been translated to *in vitro* tissue engineering and cell models.

The standard two-dimensional (2D) culturing methods do not resemble the natural environment of cells with 3D tissue configuration with complex cell – cell and cell – matrix interactions (Lou and Leung 2018). In 3D cell culture models, cell – biomaterial interactions play a crucial role in many aspects similar to 2D models. In addition, 3D models have more features to be considered when planning a suitable model, such as cell release from the matrix and nutrient flow. Thus, new materials, such as hydrogels from cellulose nanofibrils (CNF, also called nanofibrillar cellulose, or nanofibrillated cellulose, NFC) has been developed. Understanding the fundamentals, limitations, and benefits of each model is critical to their proper utilization. For this purpose, the aim of this thesis work was to detect critical ECM components and cell – biomaterial interactions in different cell culture applications and use them to induce hPSC hepatic differentiation.

This thesis introduces first, as a background, the natural ECM, how cells sense cell – biomaterial interactions and what kinds of outcome the physical, chemical, and biological cues of cell culture materials have with hPSCs. The quantitative methods to study cell – biomaterial interactions and especially AFM are introduced in the following section. After the background presentation, this thesis introduces stepwise

differentiation of hPSCs to hepatic cells fully in 3D matrix and how specific cell – biomaterial interactions can be used to induce hPSC differentiation. Also, new experimental setups to quantitatively study nonspecific and specific cell – biomaterial interactions are presented as well as how these cell – biomaterial interactions can be utilized in different 2D and 3D hPSC models.

2. Background

2.1 Interactions between cells and biomaterials

Interactions in biological systems are not different from those occurring between any other types of molecules or surfaces. The specialty in biological interactions arises from the complexity, as many types of forces and bonds are usually involved, and thus they are considered as in their own class in biophysical interactions.

Due to the complexity of the biological surfaces the interactions in biological systems are the sum of many interactions happening simultaneously and in series (Leckband and Israelachvili 2001). The high complexity arises due to large macromolecules and complex systems ranging in size from proteins to whole organs. The interactions are also dynamic and are never at thermodynamic equilibrium. These systems often undergo energy-dependent changes. Moreover, the interactions are not linear and stepwise but involve competing interactions, feedback loops, branching pathways, and regulatory mechanisms. Also, processes are not isolated; they are coupled to other reactions or interactions. Biological interactions involve a series of tightly controlled events, whose effects spread out in time and space in a regulated manner, in a manner similar to how electrical signals proceed in neurons.

Biological interactions involve both specific and nonspecific interactions and various bonding types in parallel and series. Some of these forces are short-ranged and, therefore, determine adhesion and binding energies, others are long-ranged colloidal forces that determine steering and docking (Leckband and Israelachvili 2001). The type of interactions between cells and biomaterials depends on the distance between the cell membrane and the material surface. Four different stages of interactions are commonly defined: surface recognition, early attachment, intermediate attachment (or membrane adhesion), and late adhesion (Ventre et al. 2012). Each of these occurs at a defined time and distance from the surface. The first stages of the interaction are nonspecific, and the early attachment stage initiates the stages of specific interactions.

2.1.1 Nonspecific cell – biomaterial interactions

Nonspecific interactions occur between all types of atoms, molecules, or surfaces. These interactions are spontaneous, meaning they are energy-independent. The main forces in this category are listed in Table 1 (Leckband and Israelachvili 2001). The strengths of these nonspecific physical forces between two molecules, particles, or surfaces depends on their chemistry, distance, size, and shape.

Table 1. *Most important nonspecific interaction types that can occur between cells and biomaterials (Modified from Leckband and Israelachvili 2001).*

Interaction name	Interaction type	Description
van der Waals	Usually attractive	A force that exists between all surfaces due to the interaction between three types of molecular dipoles: instantaneous, induced and permanent.
Electrostatic	Attractive if opposite charge, repulsive if same charge	A force that exists between charged molecules.
Steric	Repulsive	The short and long-range quantum-mechanical force that defines the geometry or shape of the molecule. Repulsion that arises from the compression of adsorbed polymer layers.
Electrosteric	Repulsive	Repulsion that arises from the compression of adsorbed charged polymer layers. Combination of electrostatic and steric repulsion.
Hydrogen bonding	Attractive	A special electrostatic binding interaction between positively charged H atoms and electronegative atoms, such as O.
Electrical double layer force	Attractive if opposite charge, repulsive if same charge	Osmotic force between charged surfaces due to the overlap of their electrical double layers.
Hydration forces	Repulsive	Short-range repulsion due to the formation of a hydration layer strongly attached on hydrophilic surfaces.
Hydrophobic interactions	Attractive	A special interaction in water between inert, non-polar molecules or surfaces, such as lipid bilayer of cell membranes.

Different theories and mathematical models have been proposed to describe these interactions. In particular, DLVO theory (named after Boris Derjaguin and Lev Landau, Evert Verwey and Theodoor Overbeek) combines the effects of van der Waals attraction and electrical double layer repulsion (Derjaguin and Landau 1941; Verwey and Overbeek 1948).

At the ranges of several micrometers, the biological interactions do not exist. When the distance between cell membranes and material surfaces decreases to approximately one micrometer, the surface recognition activity stage of the interactions begins (Sackmann and Bruinsma 2002; Ventre et al. 2012). This phase, that takes place within tenths of seconds, is mediated by weak nonspecific interactions that are established between the pericellular coat and material surfaces. Biological molecules, such as proteins, are usually partially charged hydrophilic molecules. For instance, protein structure is determined mostly by electrostatic interactions and hydrogen bonding. Also, the presence of hydrophilic and hydrophobic groups in the protein affect the structure in aqueous solution.

2.1.2 Specific interactions

Specific interactions arise when a unique combination of physical bonds or forces between two macromolecules act together co-operatively to form a usually strong but non-covalent bond (Leckband and Israelachvili 2001). These interactions are usually energy-dependent. Because specific interactions typically arise from a synergy of multiple bonds, they are also named lock-and-key, complementary, or recognition interactions. In biology, this is referred to as ligand – receptor interactions, which are highly dynamic. Specific interactions can further be divided into specific activated and specific non-activated interactions. ECM proteins and cell membranes have particular binding motifs responsible for these specific interactions as presented later in this thesis.

Specific interactions between two molecules or particles begin with the early attachment stage. It takes place with a time scale of seconds and at the distance of hundreds of nanometers (Ventre et al. 2012). This stage is mediated by cell membrane proteins, described in Section 2.3, that recognize specific molecular motifs of the biomaterials described in Section 2.2. Depending on the density and location of the adhesive motifs and cell membrane receptors, the cell can start to build more extensive and more stable molecular complexes to improve the adhesion to biomaterials. This intermediate attachment occurs with a timescale of tens of seconds and reduces the distance of the cell membrane from the biomaterial surface to tens of nanometers. Finally, the late adhesion phase initiates the maturation of adhesion molecular clusters that mediate a dynamic material – cytoskeleton crosstalk. The specific interactions lead to intracellular signaling cascades affecting cell behavior and fate, as discussed later in this thesis.

2.2 The extracellular matrix

All tissues consist of extracellular fluid, cells, and ECM. ECM is secreted by cells and is composed of a great variety of ECM macromolecules. The different combination, spatial organization, and immobilization of these substances give rise to various types of scaffolds for cells that characterize the different body tissues and organs. ECM macromolecules include collagens, elastic fibers, adhesive glycoproteins, glycosoaminoglycans (GAG), and proteoglycans (Figure 1). Together these materials form a physical, chemical, and biological 3D environment for cells. The natural environment of the cells needs to be known and understood before it is possible to create *in vivo*-like cell culture models.

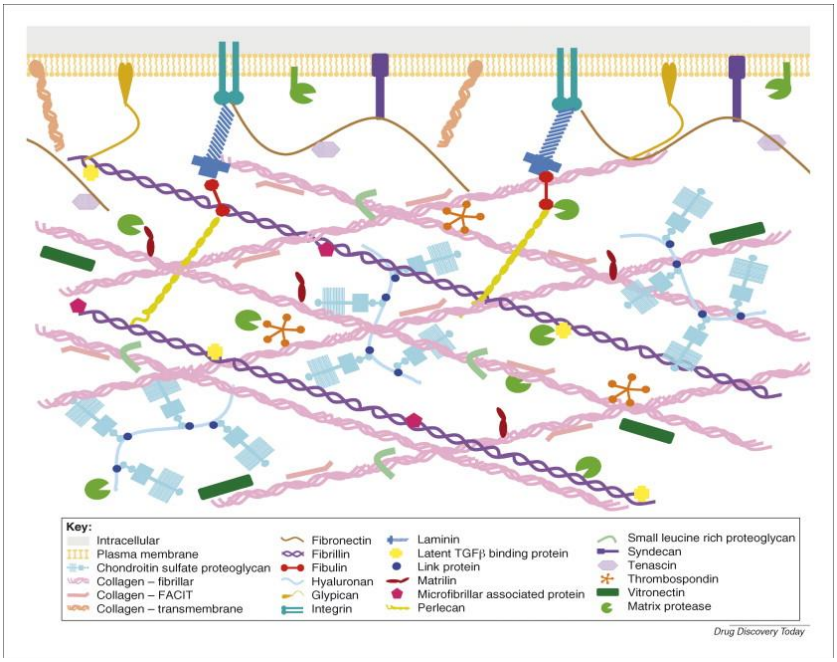


Figure 1. *Schematic illustration of the extracellular matrix (ECM), the proteins and their assembly in tissues, enzymes, and cell membrane receptors associated with cell-ECM interactions (Huxley-Jones et al. 2008, reprinted with permission from Elsevier).*

The ECM provides structural support and acts as an adhesive substrate (Hynes 2009; Rozario et al. 2010). It also provides specific signaling pathways to cells. In addition, the ECM regulates many cell functions and behavior, as discussed more comprehensively in Section 2.4. ECM has an important direct and indirect role in growth factor (GF) crosstalk with cells, such as presenting and storing GFs and cytokines with special binding sites (Hynes 2009). Through these domains, ECM regulates the nature, intensity, and duration of GF signaling (Zhu and Clark 2014). ECM proteins are often divided into structural and adhesion proteins, but this classification is simplified as some of the proteins can serve both functions.

Even though there are a large variety of ECM macromolecules, they have some common features such as large size, with molar masses of 100–1,000 kDa or more. Also, they often undergo alternative splicing, are usually extensively glycosylated, and asymmetric in shape (Engel and Chiquet 2011). In addition, all ECM proteins are multidomain proteins, in which equal or different domains are arranged in a specific domain organization. The combination of different domains makes the ECM proteins multifunctional. Degradation of ECM components have been ascribed to a family of disintegrin and matrix metalloproteinase. This degradation of ECM macromolecules often releases bioactive fragments (Reiss and Saftig 2009; Ricard-Blum and Ballut 2011).

2.2.1 Collagens

Collagens are the most abundant ECM proteins in the human body (~30% of total protein mass) (Di Lullo et al. 2002; Ricard-Blum 2011; Weissman 1969). The collagen family consists of 28 members that contain at least one triple-helical domain (Ricard-Blum 2011). Further diversity occurs due to several molecular isoforms for the same collagen type and due to hybrid isoforms. Most of the collagens assemble to complex networks. They have an important role in defining tissue structure and contribute to the shape, organization, and mechanical properties of tissues. Collagens also serve as a reservoir for GFs and cytokines (Rozario et al. 2010). Some collagens are specific for a given tissue and have a restricted tissue distribution and, hence, specific biological functions (Zhang et al. 2003; Ricard-Blum 2011).

Collagens are broadly classified into fibrillar and non-fibrillar forms. Collagen types I, II, and III are the most abundant collagens in the human body and have a fibrillar morphology (Figure 1) (Rosso et al. 2004). They are responsible for the tensile strength of the tissues. Other collagens, such as types IV, VII, IX, X, and XII are associated with collagen fibrils or assembled into the sheets or net-like structures as basal laminae. The organization, distribution, and density of fibrils and networks vary with tissue type (Rozario et al. 2010). Collagens are multidomain proteins (van der Rest and Garrone 1990). Fibrillar collagens contain one collagenous triple-helical domain (COL) while other collagen types have several of these domains. The non-collagenous domains participate in structural assembly and are responsible for their biological functions (Ricard-Blum 2011). Fibronectin type III (FNIII), Kunitz, thrombospondin-1, and von Willebrand domain are the most abundant domains. They are frequently repeated within the same collagen molecule and are also found in other ECM proteins. The growth factor binding domains bind GFs, such as Von Willebrand domain in collagen II binds transforming growth factor beta (TGF-β) 1 and bone morphogenetic protein (BMP)-2 (Zhang et al. 2007; Zhu and Clark 2014) and the cell binding domains, for instance GFOGER binds integrins α1β1, α2β1, and α11β1 (Zhang et al. 2002).

Proteolysis of collagens by matrix metalloprotease types 1, 2, 8, 9, 13, 14, 18, and 22 release the bioactive fragments of collagens (Lauer-Fields et al. 2004; Ricard-Blum 2011). These bioactive fragments, matricryptins such as endostatin and tumstatin, regulate various physiological and pathological processes in cells and tissues (Reiss and Saftig 2009; Ricard-Blum and Ballut 2011).

2.2.2 Adhesive glycoproteins

Cells adhere to the ECM mainly through the interactions with adhesive ECM glycoproteins, such as the most abundant fibronectin, vitronectin, and laminins, as well as thrombospondins, fibrinogen, entactins, nephronectin, and tenascins (Figure

1). Each of these glycoproteins has distinct functional domains or polypeptide sequences to bind specific cell-surface receptors or other ECM macromolecules such as collagens.

Fibronectin exists both as a soluble protein in plasma and as a fibrillar polymer in the ECM (Kuusela et al. 1976; Yamada and Olden 1978). It is a dimeric glycoprotein that has two identical ~240 kDa flexible covalently linked strands (Engel et al. 1981; Erickson et al. 1981). One gene encodes fibronectin and alternative pre-mRNA splicing and posttranslational modifications result in 20 variants in human fibronectin (ffrench-Constant 1995; Hynes 1985). Fibronectins consist of repeated domains, fibronectin type I, II, and III (Hohenester and Engel 2002). The cell attachment-promoting Arg-Gly-Asp (RGD) motif is a tripeptide sequence located at a $FNIII_{10}$ domain (Hohenester and Engel 2002; Ruoslahti et al. 1985). Other cell attachment sites are CS1 and CS5 with peptides such as REDV (Dufour et al. 1988; Humphries et al. 1986). These sites can be either independent or synergistic (Aota et al. 1994). Fibronectins have several GF binding domains, such as heparin II domain ($FNIII_{13-14}$) for fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (Wijelath et al., 2006; Martino and Hubbell, 2010), $FNIII_{12-14}$ domains bind most of the GFs from the same GF families and some from the TGF-β and neurotrophin families (Lin et al. 2011; Zhu and Clark 2014; Martino and Hubbell 2010).

Vitronectins are structurally and immunologically distinct from fibronectins, but they have several functional similarities, such as cell-attachment activity and ability to bind GAGs and proteoglycans (Hayman et al. 1983; Suzuki et al. 1984). Vitronectin has two closely related polypeptides with masses of 75 and 65 kDa (Hayman et al. 1982; Hayman et al. 1983; Suzuki et al. 1984). Similar to fibronectin, vitronectin can be found in its soluble form in plasma and in its insoluble form in tissues (Jenne and Stanley 1985; Collins et al. 1987). Vitronectin has similar functional sites to those in fibronectin, for instance heparin-binding sites and the same RGD tripeptide at cell attachment sites of the proteins (Suzuki et al. 1984).

Laminins are the major cell adhesive proteins of the basement membrane and among the first ECM proteins produced during embryogenesis (Yurchenco and Wadsworth 2004). They are large (400–900 kDa) glycoproteins constituted by the assembly of three disulfide-linked polypeptide chains, α, β and γ forming a cruciform shape (Figure 2.) (Timpl et al. 1979). In humans, 11 genes code for five α, three β and three γ laminin subunits that undergo posttranslational modifications (Aumailley 2005; Aumailley 2013). The combinations of the subunits give the possibility for more than 50 different laminin types, but only 16 have been found. One common and most important function of laminins is to interact with cell membrane receptors and through this interaction to regulate multiple cellular activities and signaling pathways (Aumailley 2013). Every basement membrane contains from one to several types of laminins, and this structural

diversity determines, to a large extent, the unique physiological functions of the membranes. Laminins consist of a few distinct domains, with their number, location, size, and affinity for other molecules varying from one laminin type to another. The folded α chain extension is located at the C-terminal end of the long arm (Figure 2), forming five large laminin globular (LG) subdomains (Sasaki et al. 1988; Timpl et al. 2000). These domains are responsible for the interactions with cell-surface receptors (Aumailley 2013; Timpl et al. 2000). The three laminin short arms form the N-terminus of laminins (Figure 2.) (Aumailley 2013). The separate folding of α, β and γ chains results in three types of structural domains: the laminin N-terminal, the laminin-type epidermal growth factor-like, and the laminin IV domains (Aumailley 2005). These domains of N-terminus are mainly responsible forf laminin interactions with the other ECM proteins and laminins (Aumailley 2013). Recently, GF binding domains have also been found in laminins. Ishihara et al. (2018) have shown that laminin isoforms promiscuously bind through their heparin-binding domains (HBDs) to GFs with high affinity. These HBDs are located in the LG domains and also bind to syndecan cell-surface receptors.

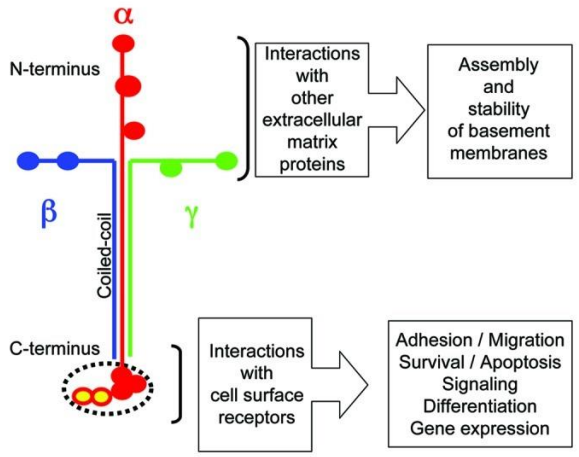


Figure 2. *The illustrative structure and the major functions of laminins. The laminin short arms (N-terminus) are involved in the interactions with other ECM macromolecules, while the end of the long arm (C-terminus) is typically involved in cellular interactions (Aumalley 2013).*

2.2.3 Glycosaminoglycans and proteoglycans

Glycosaminoglycans (GAGs) are linear polysaccharides formed by repeating disaccharide units (Jeanloz 1960; Lamberg and Stoolmiller 1974). They are negatively charged with molecular weights of roughly 10–100 kDa (Gandhi and Mancera 2008). There are two main types of GAGs; hyaluronic acid is a non-sulphated GAG, while sulphated GAGs are chondroitin sulphate, dermatan sulphate, keratan sulphate, and heparin and heparan sulphate (Gandhi and Mancera 2008; Jackson et al. 1991). They

interact with a wide range of proteins involved in physiological and pathological processes. These molecules are present on all animal ECM membranes, and some are known to bind and regulate several distinct proteins, including GFs, adhesion molecules, cytokines, chemokines, enzymes and morphogens (Gandhi and Mancera 2008). GAGs act as co-receptors for GFs of the FGF family (Gandhi and Mancera 2008; Jackson et al. 1991). These GFs need this interaction to gain their full signaling potential.

Apart from hyaluronan, all GAGs can be covalently linked to a protein backbone and give rise to the proteoglycans (Gandhi and Mancera 2008). More than 50 types have been identified, such as aggrecan, versican, and sydecans (Afratis et al. 2012; Gandhi and Mancera 2008). Proteoglycans exhibit a wide range of structural variation because of many factors, such as differences in core proteins and GAG chains. Proteoglycans are a part of ECM, but they are also present on the cell surface, such as integral membrane proteins syndecans. Virtually, all mammalian cells produce proteoglycans and either secrete them into the ECM, insert them into the plasma membrane, or store them in secretory granules. Proteoglycans have affinity to a variety of ligands, including GFs, cell adhesion molecules, matrix components, enzymes, and enzyme inhibitors.

2.2.4 Elastic fibers

Elastic fibers are ECM macromolecules having an elastin core surrounded by fibrillin-rich microfibrils (Kielty et al. 2002). The biology of elastic fibers is complex because they have various components, a multi-step hierarchical assembly, a tightly regulated developmental deposition, unique biomechanical functions, and influence on cell phenotype. Tropoelastin secreted by cells is the soluble precursor to the elastin core (Kielty et al. 2002). The core is laterally packed, thin ordered filaments (Rodgers and Weiss 2005; Pasquali-Ronchetti and Baccarani-Contri, 1997). The architecture of mature elastic fibers is complex and highly tissue specific, reflecting specific functions in different tissues. In addition to elastin, molecules such as biglycan and fibulin-1, -2 and -5 are associated in the core (Kielty et al. 2002). Fibrillin I and II form the fibrillin family and are found in the mantle of elastic fibers. Other microfibrillar core proteins are, for example, the family of the latent TGF-β-binding proteins, decorin, and microfibril associated proteins 1, 3, and 4. Several molecules localize to the elastin-microfibril interface or to the cell-surface – elastic-fiber interface such as emilins (emilin-1, -2, -3 and multimerin) and glycoproteins (Bressan et al. 1993; Doliana et al. 1999).

Matrix metalloproteinases and serine proteases are responsible for degradation of elastic fiber molecules (Kielty et al. 1994; Ashworth et al. 1999c). Elastin, tropoelastin and their degradation products can influence cell function and promote cellular responses (Rodgers and Weiss 2005). These responses include cell adhesion,

proliferation and chemotaxis. The interaction of elastin products with cells has been attributed to the elastin receptor. However, additional cell-surface receptors have also been identified. These include G protein-coupled receptors and integrins, such as $\alpha_v\beta_3$ that bind to a commonly found isoform of human tropoelastin (Rodgers and Weiss 2005).

2.3 Cell membrane receptors for cell – biomaterial interactions

Several families of cell membrane proteins mediate the interplay between cells and their environment. These proteins function as signal transducing receptors and control various intracellular pathways and further cell behavior. Some proteins are responsible for responses in environmental chemical changes or soluble factors, some form cell – cell and cell – ECM adhesion such as cadherins, CD44 and dystroglycan, integrins and syndecans (Albelda and Buck 1990; Sun et al. 2016). Many of these, such as integrins and syndecans, have multiple roles in environmental sensing of cells.

2.3.1 Integrins

Integrins are considered to be the main proteins for directing cell – biomaterial interactions (Humphries et al. 2000). Integrins are a diverse family of transmembrane proteins that consist of two subunits α and β (Figure 3). The assembly of eighteen α subunits and eight β subunits gives rise to 24 heterodimers in humans with cell-type-specific expression (Humphries et al. 2003; Humphries et al. 2006; Hynes 2002). Both subunits dictate the ligand-binding specificity. Since integrins are a part of a complex intracellular assembly of proteins, they can transmit bidirectional signals across the plasma membrane (Hynes 2002; Humphries et al. 2003; Hu and Luo 2016). They can be present either in active conformation with high affinity for extracellular ligands or inactive conformation with low affinity (Figure 3). Integrin function is regulated through multiple mechanisms, including conformational changes, protein – protein interactions, trafficking, and clustering (De Franceschi et al. 2015; Humphries et al. 2003; Kim et al. 2011; Miyamoto et al. 1995). The biological response of cells to environmental cues is strongly influenced by which integrins are expressed and active on the plasma membrane (Arjonen et al. 2012; Moreno-Layseca et al. 2019). This biological response needs a delicate balance in integrin activation controlled in a spatiotemporal manner (Bouvard et al. 2013).

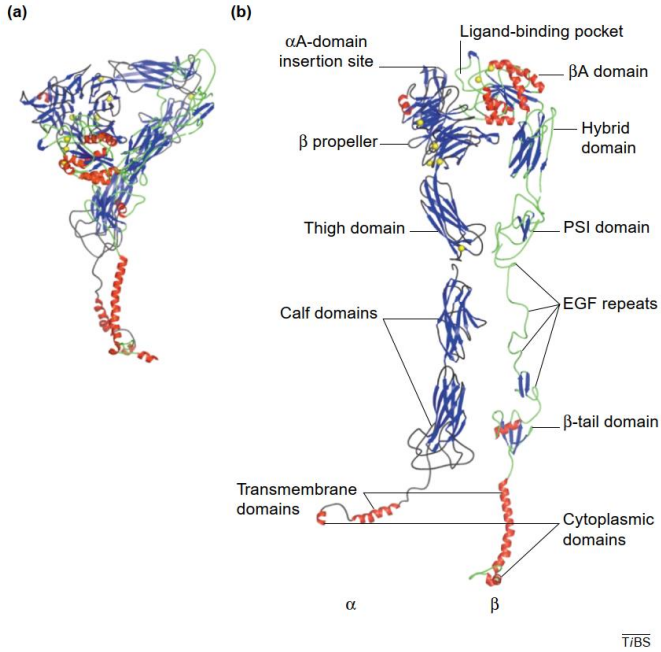


Figure 3. *Schematic representation of the structure of aVb3 integrin in non-active (a) and active (b) conformation. The α subunit is on the left, and the β subunit is on the right (Humphries et al. 2003, reprinted with the permission of Elsevier).*

The dynamic nature of integrin function requires a highly responsive receptor structure (Humphries et al. 2003). Integrins have a large extracellular domain to bind ECM, a single transmembrane helix, and a short cytoplasmic tail to link the integrin to the actin cytoskeleton of cells (Figure 3) (Humphries et al. 2003; Hynes 2002). Integrins are generally in the low-affinity state, and cell adhesion to biomaterials starts with integrin activation by the integrin conformation change, which is actively controlled by the cells (Humphries et al. 2003; Humphries et al. 2006). In addition to the ECM molecule binding domains, integrins have several other binding domains that can alter the integrin conformation and, thus, the activity, such as αA insertion site, the ligand-binding pocket, bending areas, and eight cation binding areas called metal ion-dependent adhesion sites (Figure 3) (Humphries et al. 2003). These cation binding sites are involved in ligand coordination, act as bridges between an integrin and its ligand, and possibly also stabilize the integrin structure. The binding of manganese (Mn^{2+}) and magnesium (Mg^{2+}) to their adhesion sites generally promotes the ECM molecule binding to integrins, whereas calcium (Ca^{2+}) prevents it (Humphries et al. 2003; Zhang et al. 2002). This cation function depends on cation concentration and integrin subtype. For instance, collagen I binding to $\alpha_{11}\beta_1$ integrin has been noticed to require a low μM range of Ca^{2+} ions, but is inhibited at higher, mM-range Ca^{2+}concentrations. On the other hand, $\alpha_2\beta_1$ integrin needs higher Ca^{2+}concentrations for ligand binding (Zhang et al. 2002). In addition to different conformations of

binding motifs, several complementary sites determine the ligand specificity of integrins (Humpries et al. 2003; Mould et al. 2000).

The binding of integrins to their ligands occur with low affinities in pN range (Taubenberger et al. 2007; Lehenkari and Horton 1999; Patterson et al. 2013; Rico et al. 2010). Integrins recognize specific binding motifs in their ligands as presented in Section 2.2. The extracellular domain of the integrin molecule determines the binding specificity of ECM protein ligands to integrins (Humphries et al. 2003). Most of the integrin subtypes can bind to more than one ligand type and vice versa (Huttenlocher and Horwitz 2011; White et al. 2004). For example, nine integrin subtypes can bind to fibronectin, such as types α5β1, αvβ3, and α4β1, and laminins are bound for instance by types α6β4, α3β1, and α6β1 (Humphries et al. 2006; Huttenlocher and Horwitz 2011). The subtypes binding collagens: α1β1, α2β1, α10β1, and α11β1 are titled the laminin/collagen receptor subgroup (Humphries et al. 2006; Zhang et al. 2002; White et al. 2004). This subgroup is structurally and functionally distinct with similar collagenous GFOGER motif binding domains (White et al. 2004; Zhang et al. 2003). However, they have differences in ligand-binding mechanisms, collagen subtype specificity, and cellular responses (Heino 2000; Tulla et al. 2001; Zhang et al. 2003). For instance, α1β1 prefers type IV collagen over fibril-forming collagens, opposite to the α2β1 (Tulla et al. 2001; Zhang et al. 2003). These different subtypes have different effects on cells; α1β1 signaling has been connected to cell proliferation, whereas α2β1 might regulate matrix remodeling (Heino 2000).

Several integrin subtypes can affect the activity of other subtypes through receptor cross-talk (Gonzalez et al. 2010). Integrin functions affected by crosstalk most frequently include adhesion (Calderwood et al. 2004; Pacifici et al. 1994), but also phagocytosis (Blystone et al. 1994), ECM endocytosis (Pijuan-Thompson and Gladson 1997), migration (Maubant et al. 2007), and gene expression (Huhtala et al. 1995). In addition, inside-out activating signal cross cell membrane from other cell-surface receptors, such as syndecans or growth factor receptors, increases ligand-binding affinity of integrins (Couchman and Woods 1999; Sun et al. 2016; Hu and Luo 2016). Integrin-mediated cell adhesions are highly complex processes with over ~150 different associated molecules (Huttenlocher and Horwitz 2011; Geiger et al. 2009). They appear in a variety of sizes, morphologies, and locations, depending on cell type and its environment. These adhesions are often simply called focal adhesions, but there are several subclasses. These are, for example, nascent adhesions, focal complexes, focal adhesions and fibrillar adhesions (Huttenlocher and Horwitz 2011). Ligand binding to integrins leads to the formation of a focal adhesion complex at the integrin cytoplasmic tail. Usually, two cellular activators, kindlin and talin, bind integrin cytoplasmic tails and promote the final step in integrin activation, initiating downstream signal pathways that onset different biological responses in cells (Calderwood et al. 2013). In addition to these intracellular signaling cascades, integrin clustering or aggregation is a response of integrin action to external signals (Miyamoto

et al. 2006). This integrin clustering reinforces the cell adhesion, and it occurs slowly, after 60 s contact of cells with biomaterials (Taubenberger et al. 2007).

Integrin expression varies during cell development. This variation might be due to GFs, such as TGF-β, regulating their expression (Heino 2000). Changes in integrin cassette alter cell – biomaterial interactions, affecting processes such as stem cell differentiation or cancer propagation (Huttenlocher and Horwitz 2011). Cells have matrix-induced adhesions that contain many different integrins that can affect adhesion dynamics in various ways; some subtypes are more dynamic and some more persistent.

2.3.2 Syndecans

Syndecans are a receptor family of four transmembrane heparan sulfate proteoglycans. Syndecan subtype expression is tissue and cell type specific; syndecan-4 is abundant in many cell types, while types 1, 2, and 3 are found only in some cell types (Afratis et al. 2017; Bernfield et al. 1999). Syndecans change in quantity, location, and structure during development (Bernfield and Sanderson 1990; Afratis et al. 2017; Allen et al. 2001; Bernfield et al. 1999). Also, the localization of these subtypes at the cell membrane varies (Bernfield et al. 1999). Syndecans are associated with actin cytoskeleton of cells and, thus, can regulate cell adhesion and migration. They are a link between cell – cell and cell – biomaterial interactions (Gopal et al. 2017). Both interactions with syndecans are mediated via GAG chains located at ectodomains. In addition, they interact with other cell-surface receptors, such as growth factor receptors (GFRs) and integrins, making syndecans complex and critical in many cell functions. These GAGs encode motifs, which enable direct interactions with many GFs, cytokines, chemokines, ECM macromolecules, and enzymes. In addition to actin cytoskeleton, cytoplasmic domains of syndecans have interactions with several intracellular kinases, promoting various crucial cell functions.

In addition to integrin-mediated signaling, syndecan-4 regulates the focal adhesion assembly (Afratis et al. 2017; Echtermeyer et al. 1999; Longley et al. 1999; Saoncella et al. 1999). Both syndecan-1 and syndecan-4 have direct or indirect interactions with several integrin heterodimers. For instance, integrin a5b1, require syndecan-4 as a coreceptor to mediate intracellular signaling leading to focal adhesion formation, and syndecan-1 has been associated with α6β4 integrin (Beauvais et al. 2004; Mostafavi-Pour et al. 2003).

An essential aspect of syndecans' biological role, possible also in stem cells, is their interaction with GFs and their receptors. Syndecans can act as co-receptors by binding GFs and present them to their receptors (Afratis et al. 2017). Syndecans are known to contribute in many GF signalings, such as the wingless type (Wnt) signaling pathway that is important in pluripotent stem cell maintenance and differentiation (Alexander

et al. 2000; Dravid et al. 2005). In FGF signaling, syndecans act as low-affinity receptors to which FGFs must bind to activate their high-affinity growth factor receptor and can serve as an integral subunit of the FGF receptor complex (Bernfield and Sanderson 1990; Olwin and Rapraeger 1992; Wu et al. 2001). Syndecans can, moreover, modulate the signaling properties of many growth factor families and cytokines, such as the heparin-binding growth factors, hepatocyte growth factor (HGF), and epidermal growth factor (EGF) (Afratis et al. 2017; Zhang 2010).

2.3.3 Other membrane receptors for cell – biomaterial interactions

In addition to integrins, there are also other non-integrin receptors that participate in cell – biomaterial interactions (Cloutier et al. 2019). Since they are expected to have a lower effect on cell adhesion than integrins, only dystroglycan and CD44, which have been shown to have a role in embryo development and stem cell behavior, are briefly described in this review.

CD44 is a family of polymorphic integral membrane glycoproteins broadly distributed in adult and fetal tissues. It mediates cell attachment to several ECM proteins and cell-surface ligands (Aruffo et al. 1990). CD44, also referred to as P-glycoprotein, plays a vital role in tumor progression and metastasis, especially through cancer stem cells (Morath et al. 2016). This receptor organizes signaling cascades through association with the actin cytoskeleton (Ponta et al. 2003). In normal tissues, the importance of CD44 is vital to the regulation of hyaluronic metabolism, activation of lymphocytes, and release of cytokines (Senbanjo and Chellaiah 2017). Different isoforms of this receptor are known to control stem cell maintenance and differentiation (Kim et al. 2018). CD44 interacts with a variety of ECM components, cytokines and GFs, such as hyaluronate, sulphated and unsulphated chondroitin, osteopontin, and matrix metalloproteinases (Aruffo et al. 1990; Morath et al. 2016; Senbanjo and Chellaiah 2017).

Dystroglycan has two subunits called α- and β-dystroglycan (Bozzi et al. 2009). The mucin domain of α-dystroglycan is highly glycosylated and is responsible for the binding to different ECM ligands. Dystroglycan is expressed in various tissues including muscle, the central and peripheric nervous system, as well as in many endothelia and epithelia. It plays an important role in the basal membrane assembly via its interactions with laminin and, thus, further for the deposition of other proteins of the basement membrane (Henry and Campbell 1998). It has also been showed that it is crucial for endocytic laminin-111 trafficking through its modulation of laminin endocytosis (Leonoudakis et al. 2014). Dystroglycan may play a role in the development of some cancer types (Leonoudakis et al. 2014; Cloutier et al. 2019). Laminin receptor, but not necessarily dystroglycan in the presence of suitable integrin, is required for the formation of the developmentally critical basement membrane

between endoderm and epiblast in embryo body formation from hPSCs (Henry and Campbell 1998; Li et al. 2002).

2.3.4 Integrins in hPSCs

Stem cells differentiate into somatic cells step-by-step both *in vivo* and *in vitro* (D'Amour et al. 2005; D'Amour et al. 2006), and because integrin subtypes are cell type and cell stage-specific, the integrin cassette changes during the differentiation. The identification of integrin cassette in undifferentiated and differentiated hPSCs can help to identify critical ECM components in pluripotent stem cell maintenance and directed differentiation. For instance, integrins αvβ5, α5β1, α1β1, α2β1 α1β1, α2β1, and α6β1 have been identified from hPSCs (Braam et al. 2008; Soteriou et al. 2013; Wang et al. 2015; Wong et al. 2010). Even though integrins have overlapping binding specificity, α5β1 is namely for fibronectin, αvβ5 for vitronectin, α1β1 and α2β1 for collagen, and α1β1, α2β1, and α6β1 for laminin. Among these, αvβ5 has been shown to support hPSC self-renewal (Braam et al. 2008). Different integrin subtypes and their combinations activate different intracellular signaling pathways (Gu et al. 2002; Hoshiba et al. 2016). In addition, integrin signaling can crosstalk with intracellular signaling activated by growth factors and can modulate their signaling (Comoglio et al. 2003; Streuli and Akhtar 2009).

Changes in integrin cassette during hPSC differentiation have been observed in a few studies, such as Brafman et al. 2013, Farzaneh et al. 2014, and Wong et al. 2010. For instance, the hPSC differentiation to definitive endoderm (DE), the first step towards hepatocytes, has proven to change the expression of several integrin subtypes. DE cells have been shown to highly express the integrin αVβ5 while the expression of the pluripotency-related laminin-binding integrins α3, α6 and β4 were downregulated (Wong et al. 2010). This expression profile suggested a potential role of vitronectin binding integrins in the development of DE. Also, integrin αvβ5 has been demonstrated to regulate the TGF-β signaling pathway in many cell types, including the maintenance and DE differentiation of hESC (Park 2011; Wang et al. 2015). In addition to integrin αvβ5, fibronectin binding α5β1 is upregulated in definitive endoderm cells (Brafman et al. 2013). Integrins α3βl, α6βl, and α7β1 have been speculated as possibly supporting hepatocyte-like cell differentiation (Farzaneh et al. 2014). Moreover, integrins with β1 mediate HGF and TGF-β signaling in liver development (Weinstein et al. 2001). Crosstalk between integrins and GF signaling plays a role in the differentiation process, including hepatic cell types.

It has been speculated that the culture conditions could affect the integrin presentation (Kallas-Kivi et al. 2018). The study of Wong et al. (2010) did not, on the other hand, find significant differences in integrin expression in different culture matrices of undifferentiated hPSCs nor when these cells were differentiated into definitive endoderm cells.

2.4 The human pluripotent stem cell niche

The stem cell niche is the microenvironment that covers all the elements immediately surrounding stem cells when they are in their naïve state. Stem cell fate is controlled by many factors, both intrinsic genetic and epigenetic signals and extrinsic regulators, such as GFs, hormones, and ECM components (Watt and Hogan 2000). To enable the natural hPSC niche, it is necessary to control cell interactions with other cells, ECM, and soluble factors, as well as mechanical and sometimes electrical stimuli to cells in a temporally and spatially regulated manner (Hoshiba et al. 2016). The influence of GFs on stem cell fate has gained much attention, but the role of the ECM has been relatively neglected until recently, despite the fact that the ECM is known to influence stem cell differentiation and the maintenance of stemness (Hoshiba et al. 2016; Watt and Hogan 2000; Scadden 2006). The ECM influences cellular functions through mechanical stimulation from substrates with different stiffness, regulation of soluble factor availability and activity, and intracellular signaling activated by cell adhesion molecules. Cellular functions are precisely tuned by the complex assembly of ECM molecules and not by single components (Hynes 2009). Therefore, it is necessary to clarify the comprehensive roles of both the assembled ECM as well as single ECM molecules in stem cell behavior.

2.4.1 The human pluripotent stem cell niche in maintenance

The first established culture method for hPSCs was on mouse embryonic fibroblasts (MEFs) that are mitotically inactivated (Thomson et al. 1998). Later, immortalized human placental stromal fibroblasts (ihPSFs) have also been used to avoid xenobiotics (McKay et al. 2011). These fibroblasts secrete GFs, ECM components, and cytokines into the culture media, which support hPSC pluripotency and proliferation. Depending on the cell type and age, over 70–80 extracellular/cell-surface protein types were detected in the ECM derived from CD1 MEFs and around 60 proteins in the ECM derived from ihPSFs (Soteriou et al. 2013). These proteins are, for instance, ECM proteins heparan sulfate proteoglycans, components of elastic fibers, laminin chains, fibronectin, vitronectin, and collagens I, IV, and XII, laminin-binding integrins, and some GFs (Hughes et al. 2011; Soteriou et al. 2013). It has been suggested that ECM organization plays a role in hPSC maintenance as feeders that provide the support for hPSC maintenance secrete more structural ECM components and produce a more complex fibrillar network than feeders, which do not support hPSC self-renewal (Soteriou et al. 2013). In addition, proteins that may be inhibitory to hPSC growth, such as collagens, may be overcome by the presence of crucial supportive components, such as laminin. Thus, the balance between ECM network properties and molecular composition appears critical for the support of hESC maintenance.

A culture method using Matrigel™ (Corning) secreted by Engelbreth-Holm-Swarm mouse sarcoma cells was established after the MEF culture method (Ludwig et al. 2006; Xu et al. 2001). In addition to GFs, Matrigel is composed of 1,851 unique proteins including the most abundant laminin, entactin, collagen IV, and heparan sulfate proteoglycans (Bissell et al. 1987; Hughes et al. 2010; Kleinman et al. 1982). In addition to these proteins, most of the peptides identified are structural proteins such as actin, spectrin, tubulin, and filamin. Since they are still animal-derived and poorly defined, some approaches have aimed at replacing Matrigel with purified recombinant ECM proteins.

Decellularized ECM is an alternative *in vitro* model that can elucidate the comprehensive roles of the ECM because it retains a native-like structure and composition. Decellularized ECM, also called acellular matrix (ACM), can be obtained from *in vivo* tissue or fabricated by cells cultured *in vitro* (Hoshiba et al. 2016). It is important to select the correct ACM because each type has different properties. It can be considered impossible to obtain hPSC ACM from *in vivo* tissue because of ethical issues and low ECM amounts available. The *in vitro* method has been applied with ACM derived from human feeder cells and from mouse ECS aggregates to maintain hPSC pluripotency (Abraham et al. 2010; Yan et al. 2015). Regarding mesenchymal stem cells (MSCs) it has been shown that the ability of their natural ACM to maintain cells as undifferentiated derives from the ability of ACM to activate and suppress important GF signals (Chen et al. 2007; Hoshiba et al. 2009; Hoshiba et al. 2011; Lai et al. 2010). Substrate stiffness also affects stem cell fate (Engler et al. 2006).

Although ACM includes all aspects from ECM, its composition is dependent on the cell type and cell culture conditions. The use of chemically well-defined matrices reduces batch-to-batch variability. The individual components of MEF derived ECM and Matrigel show varying levels of efficiency in supporting hPSC culture. Several proteins have been shown to function as chemically well-defined substrates. Laminin-coated surfaces are efficient in supporting the pluripotency and proliferation of hPSCs with isoforms -511 and -521, but not -111, -332, -211 and -411 (Domogatskaya et al. 2008; Miyazaki et al. 2008; Rodin et al. 2014). Collagens are also not suitable (Evseenko et al. 2009; Laperle et al. 2015; Miyazaki et al. 2008; Xu et al. 2001). The results from fibronectin are controversial (Hughes et al. 2011; Xu et al. 2001), but vitronectin has been shown to maintain the characteristics of hPSCs (Braam et al. 2008). Recombinant human laminin-511 and -521, and vitronectin are now routinely employed in well-defined hPSC cultures. This has led to the utilization of peptides found from these ECM proteins, such as laminin E8 fragments or SyntheMax (Melkoumian et al. 2010; Miyazaki et al. 2012). However, the exact mechanisms of important cell-ECM interactions in hPSC maintenance remain unclear.

Although many studies have applied exogenous ECM components to create hPSC culture substrates, relatively little attention has been given to the role of the endogenously produced ECM in hPSC self-renewal. The finding of vitronectin, laminin-511-, and -521 as suitable cell culture materials was based on trial and error and analysis of integrin subtypes found from the cells, not the analysis of the ECM components secreted by hPSC. On the other hand, the first study to analyze the ECM components from both hESCs and hiPSCs, identified α-5 laminin, including subtypes 521 and 511, as a predominant ECM component produced endogenously by both undifferentiated hPSC lines (Laperle et al. 2015). In addition, iPSC generated a bit collagen I from which it can be concluded that there are some differences in ECM profile produced by different hPSCs.

3D cell cultures resemble better the natural tissue environment with *in vivo*-like cell-cell and cell-ECM organization and polarization of the cells (Baker and Chen 2012). A good model allows the correct fate, function, and organization of hPSCs by mechanical and biochemical signaling to the cells. The 3D cell culture methods can be divided into matrix-based and matrix-free systems, where the matrix-based resembles better the natural environment of the cells. The hPSCs proliferate in 2D culture as compact colonies, and this architecture is linked to their survival, pluripotency, and self-renewal (Chen et al. 2010; Kraehenbuehl et al. 2011; Li et al. 2010). A successful 3D scaffold should be permissive to degradation by cells and migration of cells through the scaffold and should allow hPSC cell expansion without perturbing colony integrity. Also, for further analysis and applications, it would be beneficial if the formed cell spheroids could be released from the matrix. This release requires scaffold degradation in response to enzymes or other factors.

Natural ECM molecules used for hPSC expansion in 3D are for instance Matrigel, collagen, chitosan, hyaluronic acid, and alginate (Shao et al. 2015). In addition to the concern of xenobiotics with Matrigel, there are several technical problems with the use of ECM protein-based 3D cell cultures. In these systems, cell spheroids cannot be released from the scaffold without breaking the cell organization (Lou et al. 2014). In addition, the control of matrix stiffness is difficult. Synthetic hydrogels derived from polymers such as polyethylene glycol, polylactic acid, polylactic acid-co-glycolic acid, and polyglycerol sebacate has been used to culture hPSCs in 3D (Kraehenbuehl et al. 2011). These materials can be precisely tailored, but cross-linking agents needed to create 3D cast are toxic for the cells (Oryan et al. 2018).

CNF, either of plant or bacterial origin, consists of cellulose that is a linear polymer made of glucose units. These fibrils have diameters of few nanometers and lengths up to several micrometers. CNF is biocompatible and non-toxic for cells (Hannukainen et al. 2012; Pereira et al. 2013; Hua et al. 2014; Lopes et al. 2017). CNF hydrogel does not need cross-linking agents because the cellulose nanofibrils are naturally crosslinked via hydrogen bonds and physical entanglement. CNF hydrogel has shear-

thinning properties, and the stiffness can be modified by varying CNF concentration (Bhattacharya et al. 2012). This material can be modified further with chemical modifications. CNF is used in different biomedical applications, for instance, in wound healing (Jack et al. 2017; Liu et al. 2016; Rees et al. 2015; Kiiskinen et al. 2019) that has reached clinical use (Hakkarainen et al. 2016), sutures (Lauren et al. 2017) and implants (Harris et al. 2011; Modulevsky et al. 2016; Laurén et al. 2014; Nguyen et al. 2018). Chemically unmodified, xeno-free and natural wood origin CNF hydrogel, prepared by mechanical fibrillation of cellulose pulp, has been shown to support hPSCs maintenance and self-renewal in 3D (Lou et al. 2014). Also, several other cell types, such as hepatic cells, have been observed to create cell spheroids in CNF hydrogels (Bhattacharya et al. 2012). Notably, the topography of CNF scaffolds resembles natural ECM (Bhattacharya et al. 2012) and cell spheroids can be released from the hydrogel by cellulase enzyme treatment (Lou et al. 2014). Hyaluronic acid and chemically unmodified CNF do not support hPSCs cell attachment on 2D cultures, but they allow cell spheroid formation in 3D cultures. It could be speculated that integrin-cell interactions are not needed for spheroid formation and hPSCs maintenance in 3D, but this has not been confirmed with direct measurements of interactions between CNF and hPSCs or any other cell type.

2.4.2 Cell niche in stem cell differentiation

The composition of the ECM is determined by developmental and pathological conditions (Bonnans et al. 2014). *In vitro*, hPSCs need to be differentiated in a highly controlled and comprehensive manner by controlling the media and their components as well as the cell culture matrices. In regenerative medicine, incomplete differentiation may cause teratoma formation and, in drug research, mixed cell populations can give false results. *In vivo*, the cells are dynamically remodeling the ECM at each stage according to the stepwise differentiation process (Daley et al. 2008). The assembly of ECM molecules influences stem cell differentiation through orchestrated intracellular signaling activated by many ECM molecules (Hoshiba et al. 2016). As *in vivo*, also *in vitro*, the cells need altered extracellular signals along with the differentiation. Therefore, it is important to understand the comprehensive role of the ECM in stem cell differentiation as well as the functions of the individual ECM molecules.

Several interactions between cells and ECM and the signals activated by these interactions regulate cellular functions. The regulation of GF activity by binding them to ECM proteins affect the stem cell fate, not only in stem cell maintenance, but also in stem cell differentiation. The ECM can downregulate the activity of some soluble factors by binding, but sometimes the ECM can also upregulate the activity by increasing the availability of protein compared to free form (Lin 2004). As stated in Section 2.2., ECM proteins themselves can activate intracellular signaling through the interaction with cell adhesion molecules. For instance, different integrins can activate

different signaling pathways important in differentiation (Gu et al. 2002). Integrin signaling can also crosstalk with intracellular signaling activated by growth factors and modulate their signaling (Comoglio et al. 2003).

Cellular functions are precisely tuned by the combination of different ECM macromolecules (Hynes 2009). Decellularized ECM can be used for studying the comprehensive roles of ECM in stem cell differentiation similarly as in stem cell maintenance. The composition of the ECM is complex and tissue-specific, so the selection of the correct ACM is important (Hoshiba et al. 2016). Tissue-derived ACM is expected to exhibit natural mechanical properties (e.g., stiffness) and microstructure and proper regulation of GF activity by ECM macromolecule binding.

Animal-derived native tissue origin ACM or fragments of it have been used to facilitate differentiation of hiPSCs. For instance, rat liver ACM has been used to guide the differentiation of hPSCs into hepatic-like cells (Wang et al. 2016). Also, kidney, lung, and a few other tissues have been used (Batchelder et al. 2015; Du et al. 2016; Gilpin et al. 2014; McLenachan et al. 2017). Jaramillo et al. have tackled the problem of animal origin tissues by using human liver ACM to induce hepatic differentiation of hiPSCs (2018). Cell culture-derived ACM in 2D and 3D has also been used to regulate hPSC differentiation and to tackle the problems with ethical concerns or availability. For instance, Kanninen et al. have used ACM from hepatic progenitor cells to induce the hPSC differentiation to hepatic cells (2016). The composition of the ECM varies during the stem cell differentiation process. Using respective cell-derived ACM scaffolds at each maturational stage of the stem cells has shown to be successful for MSC differentiation as well as maintaining pluripotency or inducing early neural differentiation (Hoshiba et al. 2009; Hoshiba et al. 2010; Yan et al. 2015). This behavior suggests that ECM remodeling influences stem cell differentiation and that the differentiation requires tissue- and stage-specific ECM. Especially matrix stiffness and its ability to bind different GFs has shown to be critical for guiding stem cell differentiation (Engler et al. 2006; Hoshiba et al. 2009; Hoshiba et al. 2010; Hoshiba et al. 2012; Yan et al. 2015).

The use of chemically well-defined matrices reduces batch-to-batch variability. Even cell-derived ACM is a compromised model because it is challenging to obtain ACM with the composition, mechanical properties, and microstructure that are identical to *in vivo* ECM. Brafman et al. have studied hundreds of combinations of ECM proteins that induce hPSC differentiation to definitive endoderm (DE), an early embryonic cell population that gives rise to internal organs such as the lung, liver, pancreas, stomach, and intestine (2013). They have found that fibronectin and vitronectin promote differentiation through their integrins. Also, laminin has been observed to play a role in cell differentiation (Wang et al. 2015). Based on the microarray study murine ECM and cells, further differentiation towards hepatic lineage could be induced with laminin with the addition of collagen, fibronectin, and vitronectin (Flaim et al. 2005).

Interestingly, the ratio of these components impacts their efficacy in directing differentiation. However, neither the human ECM contents nor the responsible integrins have been extensively studied, so our ability to utilize integrin signaling to direct cell fate with their substrates is relatively crude.

Again, technical issues also place some limitations on cell culturing strategies, especially in 3D systems as discussed in Section 2.4.2. A good model allows correct stiffness, topography, biochemical signals, and spheroid formation, and maintenance, and release at each maturation step separately. The extracellular environment that allows correct integrin and GF signals has been shown to have great importance for stem cell differentiation. Because of the high complexity of natural ECMs, it is important to analyze the critical ECM macromolecules needed for efficient hPSC differentiation and to analyze the detailed interactions of these components with cells. It is anticipated that there cannot be one universal material suitable for all differentiation steps but tuning the interactions with hybrid materials could be a solution. The preparation of hybrid materials would require detailed studies of biomaterial – biomaterial, biomaterial – GF, and cell – biomaterial interactions.

2.5. Force measuring techniques for cell – biomaterial interactions

Diverse force measuring methods provide different types of information, such as magnitudes, timescales, or range, and can be categorized according to the type of information they provide. Some methods provide only thermodynamic data, others demonstrate the direct or indirect binding energies of molecules or particles. Indirect force measurement methods are for instance methods that provide adhesion force or minimum energy at some state of the system. These methods do not give information on the nature and range of the force or distance dependency. Direct force-measuring techniques directly measure the force between two surfaces and provide distance dependence of the force. Direct methods can provide more information about the cell – biomaterial interactions than indirect methods. However, it is challenging to exactly mimic the natural environment of the cells.

Different methods have been used to study the interaction forces and binding kinetics between cells and biomaterials, such as atomic force microscopy (AFM) (Alsteens et al. 2017; Müller et al. 2009; Taubenberger et al. 2014), microcantilever (Yamamoto et al. 1998), magnetic (Balaban et al. 2001; Kollmannsberger and Fabry 2007) or optical tweezers (Andersson et al. 2007; Neuman and Nagy 2008), micropipette aspiration (Neuman and Nagy 2008; Sung et al. 1986), reflectance interference contrast microscopy (Feng et al. 2006; Feng et al. 2010; Yin et al. 2003), total internal reflection microscopy (Lanni et al. 1985), biomembrane force probe (Ju et al. 2017), and shear flow detachment (Brown et al. 2002; Owens et al. 1988; Siddique et al.

2017) or spinning disk devices (Cargill et al. 1999; García et al. 1998a; García et al. 1998b). These methods, with their advantages and drawbacks for studying cell – biomaterial interactions are summarized in Table 2.

Table 2. *Different methods to study cell – biomaterial interactions, their advantages, and drawbacks (Leckband and Israelachvili 2001; Taubenberger et al. 2014).*

Force measuring technique	Advantages	Drawbacks
Atomic force microscope (AFM)	High sensitivity, wide force range. Force measurements can be combined with high resolution images.	Contact area and absolute separation distance between soft surfaces are not known.
Micro cantilevers	Can measure individual bond strengths (adhesion and binding forces).	Cannot measure intermolecular force-law and measures only low forces.
Magnetic tweezers	Combination with optics.	Imprecise force detection, restricted to low detachment forces.
Optical tweezers	High force sensitivity and control.	Difficult experimental setup, restricted to low detachment forces and heats the sample.
Reflectance interference contrast microscopy, total internal reflection microscopy	Very high force sensitivity.	Restricted to certain systems and only repulsive forces.
Spinning disk devices	High reproducibility and throughput, wide applicable force range.	Results difficult to interpret, not quantitative data and only analysis of a large number of cells.
Micropipette aspiration	Closely mimics biological conditions, good for adhesion forces, one of the widest range of sensitivity.	Poor resolution, accurate force-distance profiles cannot be measured, cell shape changes by aspiration.
Washing assay	Economic and simple.	Low reproducibility, no exact force quantification, only analysis of a large number of cells.
Shear flow detachment	Simple setup, useful for analysis of cells subjected to flow *in vivo* .	No exact force quantification, anly analysis of a large number of cells.
Centrifugation assay	High reproducibility and large applicable force range.	Only single detachment force per run and only analysis of a large number of cells.
Biomembrane force probe	High resolution, good temporal control.	Cell shape changes by aspiration.

Some critical parameters need to be considered while performing direct force measurements. The three most important parameters are the surface geometry, the force function, and the elastic modulus of the force-measuring spring. While the principle of direct force measurements is, generally, straightforward, the interpretation of the data may be challenging. The data obtained is usually presented as force-distance curves (Figure 4).

The range and strengths of the interaction energies, adhesion forces, and particle size determine the most suitable detection method. In the case of biological studies, the method should allow the studies in physiological conditions. Micropipettes, magnetic, and optical tweezers have disadvantages either because of low force resolution or a narrow range of detectable forces (from 10 pN to 1 nN) (Friedrichs et al. 2013). AFM-

based force spectroscopy offers the broadest range of detectable forces from 10 pN to 100 nN, with a high spatial resolution (Müller et al. 2009; Taubenberger et al. 2014).

2.5.1 Atomic force microscopy in cell – biomaterial interaction studies

In addition to the widest detectable force range, the AFM provides accurate temporal (~0.1 s to > 10 min) and spatial (~ 1 nm to ~ 100 µm, depending on the scanner and AFM model) control during the adhesion measurement at physiological conditions (Neuman and Nagy 2008; Taubenberger et al. 2014). The AFM can be used to measure the interaction forces between a substrate and a probe, which typically consists of a molecule, a tip, a bead, or a cell attached to a cantilever. The AFM can detect forces from single receptor-ligand bonds (~60–80 pN) to those covering the adhesion of entire cells (>>1 nN). However, spring or cantilever stiffness affects both the force sensitivity and the force range. The stiffer the cantilever, the lower the sensitivity is. On the other hand, stiffer cantilevers allow probing stronger forces.

AFM instruments employ, for instance, mechanical, piezoelectric, or optical means for moving and controlling the cantilever base or substrate surface and for measuring cantilever deflections (Leckband and Israelachvili 2001). Usually, a laser beam and a detector are employed to measure the deflection of the cantilever (Figure 4). AFM is sometimes combined with various optical techniques, such as fluorescence microscopy. AFM is not an invasive method, meaning the measurements can be performed without permanently disturbing the cells.

While recording the surface topography, the AFM can be employed to detect adhesion and elasticity. The elasticity of the interacting material can be calculated from the slope of the approach curve when material and probe are in contact. The deformation of elastic bodies can be explained by the Hertz theory for nonadhering surfaces and Johnson-Kendall-Roberts theory for adhering surfaces (Leckband and Israelachvili 2001). On the other hand, adhesion forces and energies can be calculated from the force-distance curves on retraction (Figure 4). The adhesion can be described with two parameters: the pull-off force or detachment force, which is the maximum force needed to separate the two surfaces from contact, and the adhesion energy, which can be calculated from the area between the retracting force curve and the x-axis as shown in Figure 4.

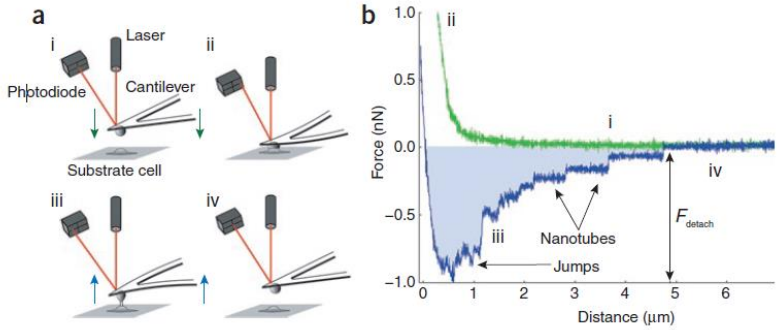


Figure 4. *AFM-based SCFS method (a), where the surfaces are brought into contact (i) and allowed to adhere (ii) before separation (iii and iv). Obtained unnormalized force-distance curve (b), where maximal detachment force is described as F_{detach} and adhesion energy the area between curve and x=0 (Müller et al. 2009, reprinted with the permission of Springer Nature).*

From the force curves, it is also possible to distinguish different types of interactions between cells and biomaterials. It could be concluded from the force-curve profile if the interactions are mediated by receptors associated to the cytoskeleton or receptors without association to cell cytoskeleton (Krieg et al. 2008; Müller et al. 2009; Sun et al. 2005a). The unbinding of the bonds with receptors attached to the cell cytoskeleton can be seen as jumps in force-distance curves (Figure 4b). Activated integrins, syndecans and CD44 receptors are linked to cell cytoskeleton as presented in Section 2.3. as well as cadherin, IgCAMs and selectins (Juliano et al. 2002). Cytoskeleton independent interactions have a stair-like force profile of nanotubes or tethers (Figure 4). Sun et al. (2005a) have not found any differences in tether forces when probing different cells with uncoated, collagen I coated, not concavalin-A coated cantilevers. Thus, it seems likely that nonspecific cell – material interactions often have an unbinding profile of tethers in force-distance curves.

AFM force spectroscopy has been applied to study the interactions between biomaterials and human cells, such as myeloid leukemia (Li et al. 2003), HeLa (Friedrichs et al. 2010), breast cancer (Taubenberger et al. 2013), mesenchymal stem (Bertoncini et al. 2012), and embryonic kidney cells (Yermolenko et al. 2010). These measurements were performed with AFM-based single cell force spectroscopy (SCFS) by attaching a cell to the AFM cantilever and then probing the cell against the materials attached to the substrate. This method has been used to examine the contribution of adhesion to some biological and medically relevant processes. These include some mechanisms in cancer (Fierro et al. 2008) and immune cell adhesion (Wojcikiewicz et al. 2003; Wojcikiewicz et al. 2009), as well as cell adhesion and migration in development (Krieg et al. 2009; Puech et al. 2005; Ulrich et al. 2005). To the best of our knowledge, the adhesion between human pluripotent stem cells and

biomaterials of any type have not been demonstrated previously in the literature using AFM or other quantitative methods.

AFM has been used to study the binding of integrins to their ligands. Studies performed with isolated integrins suffer from the lack of activating inside-out signaling of the cells to integrins. It has furthermore been shown with SCFS that the context of the adhesive sequence within an ECM protein has considerable influence upon the final binding force for receptor interaction (Lehenkari and Horton 1999). Thus, studies performed with only an isolated adhesive sequence, such as RGD, are not as such adequate for understanding the *in vivo* interactions.

The role of single integrin subtype to the interactions of rat vascular smooth muscle cell with fibronectin (Sun et al. 2005b) or human HeLa cells with fibronectin and collagen I (Friedrichs et al. 2010) has been studied by blocking specific integrins with antibodies or peptides (Sun et al. 2005b). While this study design gives us valuable information about which integrin subtypes are involved in cell adhesion with studied material, it also has limitations. Firstly, antibodies also interact with biomaterials, and this might affect the recorded adhesion. Secondly, as presented in Section 2.3, many ECM proteins have overlapping specificity to several integrin subtypes. For instance, Dao et al. (2013) have pointed out that one inhibitory antibody for laminin-specific integrin subtype did not completely block the specific interactions between Chinese hamster ovary cells and laminin. It is not possible to block all the cell adhesion receptors at the same time to study the nonspecific cell – biomaterial interactions. On the other hand, the role of integrin activation by divalent cations (Friedrichs et al. 2010; Lehenkari and Horton 1999; Patterson et al.; Trache et al. 2010), adhesion regulation (Friedrichs et al. 2007; Friedrichs et al. 2008; Tulla et al. 2008) and maturation by integrin clustering (Friedrichs et al. 2010; Taubenberger et al. 2007) has been tested in a few studies. Receptor crosstalk, where the binding of one integrin type (the transducer) alters the behavior of a different integrin type (the target) on the same cell has also been studied (Friedrichs et al. 2010).

Although being a highly promising tool with demonstrated usefulness to study many processes involved in cell – biomaterial interactions, SCFS studies also have several limitations and challenges. For instance, primarily animal-derived model cells, such as Chinese hamster ovary cells or Madin-Darby canine kidney cells have been used. These cells are distinct in terms of receptors, enzymes, and functions from human cells used for drug toxicity testing or clinical applications (Burkina et al. 2017; Williams 2018). Some of the studies have also been performed at room temperature and not physiological conditions. In addition, this method can be relatively harsh for the cells, the cell viability is difficult to control, and the cell is not at a native stage lacking *in vivo*-like polarization. For hPSC-biomaterial interaction studies, SCFS cannot be employed since these cells do not survive as single cells. Also, neither tissue samples nor 3D cell spheroids can be studied with this method. The solution could be AFM-

based colloidal probe microscopy (CPM) (Ducker et al. 1991). In this system, cells are attached on the substrate and the biomaterials are deposited on spherical microparticles attached at the free end of cantilevers (colloidal probes).

Previously, only a few publications have reported cell adhesion studies where material was functionalized on AFM tips and cells were located on a substrate (Krieg et al. 2009; Lehenkari and Horton 1999; Sun et al. 2005a; Sun et al. 2005b). Lehenkari and Horton (1999) have faced tip contamination and have speculated that this study design could cause contamination on the tip from the cells and further disturb the analysis. This contamination problem has been considered to apply also to AFM-based CPM, and, therefore, mostly SCFS has been used. On the other hand, highly adhesive biomaterials could also detach from the substrate and attach onto the cells in SCFS. Hence, both CPM and SCFS requires careful control of sudden changes in the force profiles to avoid artefacts due to the material transfer.

The contact area has a great impact on the magnitude of recorded adhesion in addition to applied force because the receptor amount varies with the contact area. It is not possible to directly measure the actual contact area in CPM because of the soft environment and the, often, not transparent cantilever, but it is proportional to the size of the probe. Hence, forces obtained in CPM experiments are usually normalized by the probe radius, to facilitate the comparison between data obtained with different colloidal probes (Ralston et al. 2005). The force normalization is often done by $F/2\pi R$ in different publications because that magnitude corresponds to the interaction energy per unit of area between two flat surfaces according to Derjaguin's approximation (Israelachvili 2011). Nevertheless, normalization by F/R is more common in the literature (Ralston et al. 2005). Unlike CPM studies between non-cellular materials, cell adhesion studies usually present their results without any normalization, which makes the comparison and interpretation of the results from different studies more difficult. Although a contact area-dependence of cell adhesion is also expected in SCFS experiments, it is interesting to note that Dao et al. have not observed a correlation between contact area and maximum detachment force when studying the adhesion of Chinese hamster ovary cells to different materials (Dao et al. 2013).

Although there are some deficiencies in the current cell – biomaterial adhesion studies performed by AFM, there is a lot of useful information already available regarding research methods, the roles of integrin subtypes, and integrin activation mechanisms that can be utilized in hPSC studies.

3. Aims

The overall aim of the thesis work was to quantitatively study the adhesion of cell lines, intensively used in pharmaceutical research (human hepatocarcinoma cells and especially human pluripotent stem cells) with relevant biomaterials (especially laminin-521 and cellulose nanofibrils), to detect critical ECM macromolecules, soluble factors, and cell – biomaterial interactions in different cell culture techniques and applications, and finally use them to increase the functionality of hPSC-based cell models in 2D and 3D.

The specific aims of the thesis were:

1. To test whether AFM-based CPM is a suitable method to probe cell – biomaterial interactions (Publication I).
2. To find out whether there is a correlation between cell behavior *in vitro* (cell attachment and growth on biomaterials) and cell – biomaterial force measurements by AFM-based CPM (Publications I, II).
3. To determine the magnitude and type of the adhesion between chemically unmodified CNF and cells (Publications I, II).
4. To evaluate quantitatively nonspecific and integrin-mediated specific interactions between cells and biomaterials, analyzing the effect of integrin activation, cell viability and presence of divalent cations on such interactions (Publication II).
5. To detect the main ECM proteins of acellular matrix from hepatic progenitor cells and to test what integrins are present at different hPSC differentiation stages towards hepatic lineage (Publication III).
6. To see if it is possible to induce hPSCs differentiation from definitive endoderm cells to hepatocytes with the detected key ECM proteins and to identify the critical components for inducing the differentiation (Publication III).
7. To test if unmodified CNF hydrogel is a suitable material for stepwise hepatic differentiation in 3D (Unpublished data IV).

4. Overview of the materials and methods

The materials and methods used in this thesis are briefly presented in this chapter and summarized in Table 3. For more detailed information on the methods, the reader is referred to the appended manuscripts.

Table 3. *Overview of the materials and methods*

Method	Study	Target	Publication
2D cell culturing	Cell behavior on materials	HepG2, WA07, H9-GFP, iPS(IMR90)-4	I, III
2D cell culturing	Hepatic differentiation	H9-GFP, iPS(IMR90)-4, WA07	III
3D cell culturing	hPSC spheroid formation, Hep	iPS(IMR90)-4, WA07	Unpublished results
AFM imaging	The morphology and integrity of biomaterial coating	Col I, Col IV, LN-521, CNF	I
Albumin ELISA	Cell functionality	H9-GFP, iPS(IMR90)-4	III
AFM-based colloidal probe spectroscopy	Cell-biomaterial interactions	HepG2, WA07	I, II
Confocal microscopy	Cell characterization, Characterization of the ECM components	HepaRG, H9-GFP, iPS(IMR90)-4, WA07	III
Conventional PCR	Characterization of the ECM components	HepaRG	III
CYP activity measurement	Cell functionality	iPS(IMR90)-4	III
CYP induction	Cell functionality	iPS(IMR90)-4	III
Field emission scanning electron microscopy (FESEM)	The morphology and integrity of biomaterial coating and AFM tip characterization	AFM tip, LN-521, CNF	II
Immunostaining	Cell characterization, Characterization of the ECM components	HepaRG, H9-GFP, iPS(IMR90)-4, WA07	III
Live/dead staining	Cell viability	iPS(IMR90)-4	Unpublished results
Phase contrast microscopy	Cell characterization	HepaGR, HepG2, H9-GFP, iPS(IMR90)-4, WA07	I, II, III
qPCR	Cell characterization	HepaGR, HepG2, H9-GFP, iPS(IMR90)-4, WA07	III
Scanning electron microscopy (SEM)	The cell surface morphology	HepG2, WA07	II
Trypan blue exclusion test	Cell viability	HepG2, WA07	I

4.1 Biomaterials

All biomaterials used are presented in Table 4. They are all commercially available and xenobiotic-free. Laminins (LN), fibronectin (FN), and collagens (Col) are ECM proteins, CNF is a wood-derived glucose-based biomimetic material.

Table 4. *Summary of biomaterials used and the substrate coating methods*

Biomaterial	Producer	Concentration	Coating method
Human recombinant laminin 511 (LN-511)	Biolamina	20 µg/mL	Immersion
Human recombinant laminin 521 (LN-521)	Biolamina	10-20 µg/mL	Immersion
Human fibronectin (FN)	Sigma-Aldrich	25 µg/mL	Immersion
Human collagen I (Col I)	Sigma-Aldrich	1 mg/mL	Spin-coating
Human collagen IV (Col IV)	Sigma-Aldrich	1 mg/mL	Spin-coating
Nanofibrillar cellulose (NFC, CNF)	UPM-Kymmene	<0.875 %	Immersion or spin-coating

4.2 Cell cultures

All cells used were human-derived and well established. Cells were cultured at 37°C in a humified tissue culture incubator with 5% CO_2.

4.2.1 Human liver cell lines

HepaRG cell line (Gripon et al. 2002) was obtained from Biopredict (Saint-Grégoire, France). These cells were cultured in William's E medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 50 IU/ml penicillin, 50 µg/mL streptomycin, 5 µm/mL insulin, 1 mM L-glutamine, and 50 µM hydrocortisone. To promote hepatic maturation, the HepaRG cells were cultured for two weeks before use with additional dimethyl sulfoxide (DMSO) (Gripon et al. 2002). Human hepatocellular carcinoma HepG2 cells were purchased from ATCC (HS-8065; Knowles et al. 1980). They were maintained in DMEM with high glucose and pyruvate content (Gibco) supplemented with 10% FBS.

Both HepaRG and HepG2 cells were cultured in 75 cm^2 cell culture flasks. HepaRG cells were passaged every two weeks and HepG2 cells twice a week with the well-established protocols or as described by us (Cerec et al. 2007; Gripon et al. 2002; publications I and II).

4.2.2 Human primary hepatocytes

The frozen human hepatocytes (BD Biosciences, lot 99 and 95) were recovered by using a cryopreserved hepatocyte purification kit (BD Biosciences) according to the manufacturer's instructions. The cells were used as controls in conventional PCR and qPCR.

4.2.3 Human pluripotent stem cells

The hESC lines WA09 (also known as H9 cells) and WA07 (Thomson et al. 1998) and hiPSC line iPS(IMR90)-4 (Takahaski et al. 2007; Yu et al. 2007) were purchased from WiCell Research institute. The H9 cells were further genetically modified to H9-GFP cells by Lou et al. (2014). The cells were cultured on Matrigel-coated (BD Biosciences) well plates. The mTeSR™1 medium was renewed daily. After removing the differentiated cells manually with aspiration pipette, the cells were passaged. The cell passaging was performed when cell confluency reached 70% by using Versene 1:5000 (Invitrogen) for WA07 and iPS(IMR90)-4 cells and Dispase (STEMCELL™ Technologies) for H9-GFP cells.

4.2.4 Hepatic differentiation of human pluripotent stem cells in 2D

The stepwise differentiation protocol used for hPSC induction to hepatic cells followed the embryo development stages (Figure 5). The differentiation media used were modified from well-established protocols (D'Armour et al. 2005; Hay et al. 2008; Si-Tayeb et al. 2010; Toivonen et al. 2013; Kanninen et al. 2016). The hPSCs were first induced to DE cells, seeded on ECM proteins, and then stepwise differentiated to hepatic cells.

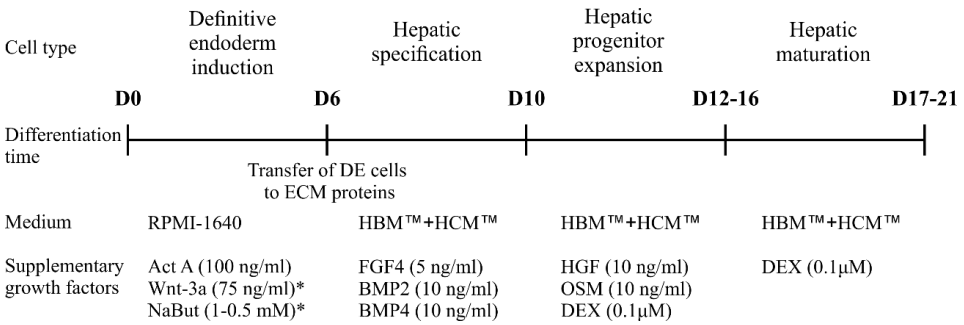


Figure 5. *Differentiation protocol of human pluripotent stem cells to hepatic-like cells in vitro. After the stem cells were induced to definitive endoderm (DE) cells, they were transferred to extracellular matrix (ECM) proteins and the differentiation was continued with Hepatocyte culture medium (HBM basal medium with HCM™ SingleQuots™ Kit without rhEGF and gentamicin-amphoterin-1000, Lonza) with stage-specific growth factors. *Components only used with H9-GFP cells. Act A, Activin A; Wnt, Wingless type; NaBut, Sodium butyrate; FGF, Fibroblast growth factor; BMP, Bone morphogenetic protein; HGF, Hepatocyte growth factor; OSM, Oncostatin M; DEX, Dexamethasone.*

4.2.5 Hepatic differentiation of human pluripotent stem cells in 3D

The stepwise 3D differentiation protocol used for hPSC induction to hepatic cells followed the protocol from publication III (Section 4.2.4). The hPSC spheroid formation in CNF hydrogel and release with cellulase enzyme (UPM-Kymmene) has

been published by Lou et al. 2014. After spheroid release, the differentiation started either in CNF hydrogel (Figure 6) or in suspension culture (Figure 7).

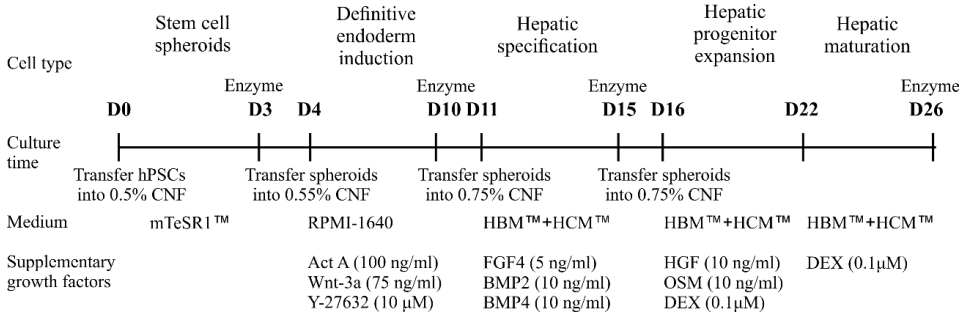


Figure 6. *Differentiation protocol of human pluripotent stem cells to hepatic-like cells in vitro. The stem cells were seeded in cellulose nanofibrils (CNF) hydrogel to form spheroids. After the hydrogel degradation with enzyme treatment, the cells were induced to hepatic cells with similar steps in new hydrogel environment. Act A=Activin A; Wnt=Wingless type; FGF=Fibroblast growth factor; BMP=Bone morphogenetic protein; HGF=Hepatocyte growth factor; OSM=Oncostatin M; DEX=Dexamethasone.*

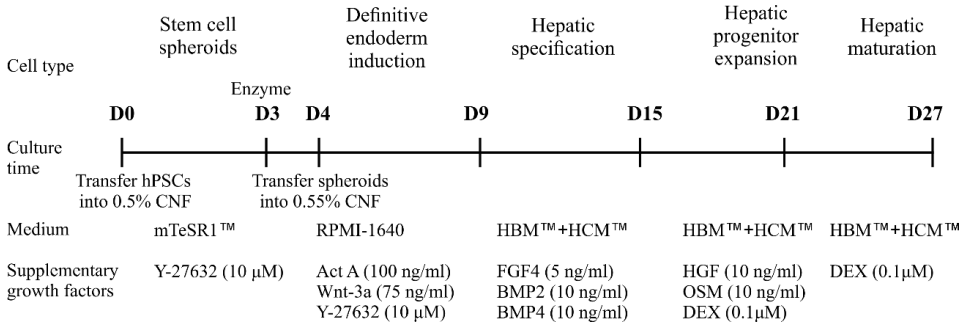


Figure 7. *Differentiation protocol of human pluripotent stem cells to hepatic-like cells in vitro. The stem cells were seeded in cellulose nanofibrils (CNF) hydrogel to form spheroids. After the hydrogel degradation with enzyme treatment, the cells were induced to hepatic cells in suspension environment. Act A=Activin A; Wnt=Wingless type; FGF=Fibroblast growth factor; BMP=Bone morphogenetic protein; HGF=Hepatocyte growth factor; OSM=Oncostatin M; DEX=Dexamethasone.*

4.3 Analysis methods

4.3.1 Cell viability

Cell viability was monitored with the phase contrast microscope. During force measurements the viability was monitored with a digital camera (uEye capture device filter with camera model UI148XLE-C) connected to the AFM instrument. After the AFM spectroscopy force measurements, cell viability was tested with Trypan blue exclusion test with the protocol modified from Perry et al. (1996) as presented in Publication I.

LIVE/DEAD® Viability/Cytotoxicity kit (Invitrogen) was used to detect cell viability in spheroids after hepatic differentiation. The dye was incubated for 30 minutes before imaging with confocal microscope.

4.3.2 Gene expression

Total RNA was extracted from cells with TRIzol reagent (Invitrogen) or RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The RNA concentrations were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The RNA was converted to cDNA with High Capacity RNA-to-cDNA kit (Applied Biosystems). The cDNA samples were used in conventional PCR and qPCR.

Conventional PCR was used to study the gene expression of the ECM proteins in HepaRG cells. We used KAPA HiFi HotStart kit (KAPA Biosystems) and the PCR cycles were performed on a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories). The PCR cycling conditions are presented in Publication III. The PCR products were examined by standard agarose gel electrophoresis and visualized under a UV transilluminator (Syngene Genius Bio Imaging System, Synoptics) as described in Publication III. The size of the PCR products was assessed by comparison with a base pair ladder (O'GeneRulerTM Low Range DNA Ladder, SM1203, Fermentas).

Quantitative PCR was performed with StepOnePlus Real-Time PCR System (Applied Biosystems) using Fast SYBR Green Master Mix (Applied Biosystems) or TaqMan Universal Master Mix II (Applied Biosystems). Housekeeping gene ribosomal protein, large, P0 (RPLP0) served as an endogenous control. The primers were synthetized by Oligomer Oy (Helsinki, Finland). The relative mRNA expression was calculated by using relative standard curve as presented in Publication III.

4.3.3 Protein expression

Direct immunofluorescence staining was used to analyze protein expression of the cells. The cells were fixed with 4% paraformaldehyde as described in the publications. After fixation the cells were permeabilized with 0.1% Triton X-100 or 0.5% Saponin if needed and blocked with 10% normal goat or donkey serum. The primary antibodies

were incubated with the cells overnight followed by the incubation of secondary antibodies conjugated with Alexa Fluor 594 (Invitrogen). Cell nuclei were stained with DAPI (Sigma-Aldrich) or SYTOX green (Invitrogen). Confocal imaging was performed as described in Section 4.3.5.

4.3.4 Cell functionality

Secretion of human albumin from the cultured cells was determined with Human Albumin ELISA Quantification Set (Bethyl Laboratories) according to the manufacturer's protocol. The cells were lysed with RIPA buffer (ThermoFisher Scientific) with protease inhibitor cocktail (Sigma-Aldrich) as described in Publication III. The amount of the protein was measured with a Pierce BCA Protein Assay Kit (ThermoFisher Scientific) and normalized with the total protein content.

The CYP3A4 activity was measured with P450-GloTM CYP3A4 assay (Promega) containing luciferin isopropyl acetate (luciferin-IPA) with the protocol provided by the manufacturer. Luminescence was recorded with a plate reader (Varioskan Flash, ThermoFisher Scientific).

The inducibility of CYP3A4 and CYP3A7 enzymes in the cells was studied with either of the known inducing substrates: dimethyl sulfoxide (DMSO), dexamethasone (DEX), phenobarbital, or rifampicin. The analysis was performed with P450-GloTM CYP3A4 or with qPCR (see Section 4.3.2).

4.3.5 Cell – biomaterial interactions

Cell – biomaterial interactions with living and dead cells were quantitatively probed with AFM force spectroscopy or AFM-based CPM. The AFM used was a MultiMode 8 AFM with a NanoScope V controller (Bruker) equipped with a PicoForce scanner. The cells cultured on a substrate were placed in the AFM liquid cell and the biomaterials were adsorbed either on colloidal probes attached on tipless cantilevers or on AFM cantilever tips with the coating methods presented in Section 4.1. and publications I and II. Cells were probed at different locations and contact times (1, 10, and 30 s) in 1 x PBS+ or 1 x PBS- medium. The macromolecules in cell culture media, such as growth factors, could disturb the experiments so the media were kept as simple as possible. The approach and retraction velocities were 2 μm/s. The maximum applied normalized force was in the range of 0.25–0.40 nN for the experiments with the special probes of 65 nm contact area, whereas, for the experiments with the colloidal probes, the maximum applied force (F/R, where R is the radius of the probe) was 0.15–0.8 mN (typically around 0.6 mN/m). Because the applied pressure is determined by the applied force and the contact area, lower forces were applied with the special probes because these probes had smaller cell – probe contact area than colloidal probes. The experiments were carried out at +37°C for living cells and at room temperature for dead cells. The detailed setups are presented in Publications I

and II. The number of measured and analyzed force curves for each system are presented in the supplementary tables in Publications I and II.

The obtained force curves were analyzed further with AFM Force IT software (ForceIT) and Origin Pro (OriginLab Corporation) softwares. The adhesion energies and detachment forces were calculated and, when measured with colloidal probes, the forces were normalized with the equation: normalized force = F/R, where F is the unnormalized force, and R is the radius of the colloidal probe. The radii of the used probes are presented in the supplementary tables in Publications I and II.

4.3.6 Imaging

The cell morphology, growth and viability were followed with phase contrast microscopes (Leica DM750 and Leica DM II LED). Pictures were captured with LAS EZ software (Leica Microsystems).

Fluorescence microscopy was performed with Leica TCS SP5 II HCS A confocal microscope as presented in Publication III. DAPI was excited with a UV (diode 405 nm / 50 mW), SYTOX green as well as live/dead dyes Calcein AM and Ethinidium homodimer-1 with an argon laser (488 nm / 35 mW), and Alexa Fluor 594 with a (561 nm / 20 mW) laser. Emission was acquired with PMT band HyD detectors. The images were analyzed with Imaris software (Bitplane). Immunofluorescence of the H9-GFP cells and their derivates were imaged with a Zeiss Axioplan microscope.

AFM imaging was used to check the integrity and morphology of biomaterial coatings on colloidal probes. The AFM was the same one as used to study cell – biomaterial interactions, but an E scanner and ScanAsyst mode were used to acquire the images in air. The images were further analyzed with NanoScope Analysis 1.5 Software (Bruker).

Field-emission scanning electron microscopy (FESEM; Zeiss Sigma VP) was used to visualize the AFM tips structure. AFM tips were mounted on double sided carbon tape fixed on the FESEM metal stubs. Fiji ImageJ software (Research Services Branch, NIH, Bethesda) was used for the image analysis.

Scanning electron microscopy (SEM; FEI Quanta series) was used to visualize the HepG2 and WA07 cell surface. Silica bioreplicas from the cells were prepared as presented earlier (Lou et al. 2015) and mounted on borosilicate cover glasses or silicon substrates and sputter-coated with Au/Pd.

4.3.7 Statistical analysis

For publications I and II statistical significance was determined with OriginPro software by using Welch's *t*-test. Differences of $p \leq 0.05$ were considered significant. Standard error of mean was used to describe the error in force curves and standard

deviation in image analysis. For image analysis, mean values of the root mean squared surface roughness values were used to describe the surface roughness of biomaterial films.

For publication III and unpublished data statistical significance was determined by one-way ANOVA with SigmaPlot 11.0 software. Differences of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered to be significant. To describe error, standard deviation was used.

5. Summary of the main results

The main results of this thesis are briefly presented below and more comprehensively in original Publications I-III and their supplements. The results are discussed in Chapter 6.

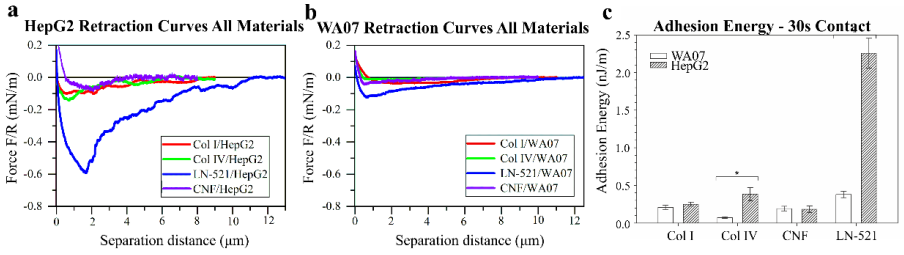
5.1 Quantitative cell – biomaterial interactions explain the cell behavior in different *in vitro* cell models

The design of *in vivo*-like cell culture models should take into account how the cells interact with the surrounding materials and how these interactions affect cell behavior. Different applications and culture methods need dissimilar signals and interactions from the matrix to the cells. AFM has shown to be an excellent method for these studies, but the commonly applied SCFS is not suitable to probe hPSCs that cannot survive as single cells. In addition, more *in vivo*-like 3D cell spheroids or tissue samples cannot be examined with SCFS because they are too large to be used as probes. Thus, we tested whether AFM-based CPM could be applied for the studies.

Using the coating methods described in Publication I and in Nugroho et al. (2019), we successfully produced evenly spread and stable biomaterial layers onto the glass probes with human recombinant LN-521, chemically unmodified CNF, human Col I and human Col IV. Unlike all the other tested materials, CNF is the only material that is not a protein and does not naturally exist in human ECM. Col I and CNF have fibrillar morphology, while LN-521 and Col IV do not form fibrils. All these materials have previously been used with the tested cell lines in different *in vitro* cell culture models. In addition to the hPSC line WA07, we used well-established hepatocarcinoma HepG2 cells that are widely used in drug toxicity testing. These cell lines have distinct properties in terms of gene and protein expression as well as behavior, and that was reflected in the force spectroscopy results presented below.

Our studies showed that CPM is a suitable method to study cell – biomaterial interactions (Publication I). It should be noted that the colloidal probes used have similar sizes to cells, and thus the unnormalized values could be compared to previously reported results from SCFS studies. We recorded distinct adhesion behavior between the two cell lines (Publications I and II). Generally, the aggressively spreading nature of HepG2 cells resulted in stronger adhesion forces with the tested biomaterials compared to the delicate WA07 cells (Figure 8). The strongest adhesion of both cell types was observed to LN-521. Collagens showed long-range pull-off forces but moderate adhesion energy with HepG2 cells, but low adhesion with WA07 cells, as could be expected based on their integrin cassette (Publications I). Both cell types showed negligible adhesion to CNF. Also, unlike all the other tested materials,

the cell adhesion to CNF was not contact-time dependent. A comparison of the force data with cell behavior *in vitro* indicated that the adhesion energy was the parameter that best correlated with the *in vitro* cell adhesion on the materials. The successful growth of the cells on a biomaterial required cell – biomaterial adhesion energies above 0.23 nJ/m. The adhesion values are presented in more detail in the supplementary data of Publication I, including normalized and unnormalized values.



d **Summary - HepG2 30 s contact**

	Number of force curves analyzed	Normalised adhesion energy (nJ/m)	Adhesion energy (fJ)	Normalized maximum pull-off force (mN/m)	Maximum pull-off force (nN)
Col I	22	0.249 ± 0.03	3.9 ± 0.4	0.074 ± 0.06	1.16 ± 0.9
Col IV	11	0.384 ± 0.09	5.27 ± 1.2	0.09 ± 0.01	1.23 ± 0.2
CNF	26	0.182 ± 0.04	2.68 ± 0.6	0.045 ± 0.01	0.66 ± 0.2
LN-521	18	2.256 ± 0.2	32.9 ± 2.9	0.482 ± 0.04	7.04 ± 0.5

e **Summary - WA07 30 s contact**

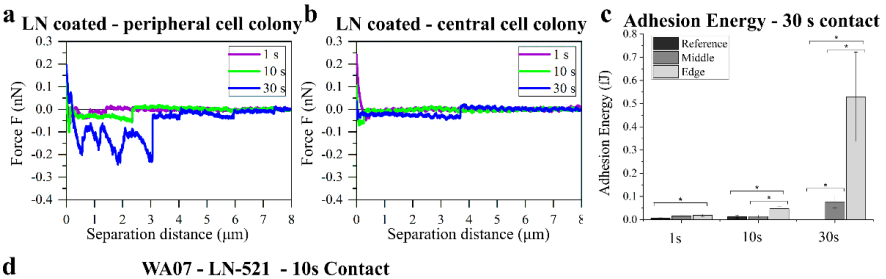
	Number of force curves analyzed	Normalised adhesion energy (nJ/m)	Adhesion energy (fJ)	Normalised maximum pull-off force (mN/m)	Maximum pull-off force (nN)
Col I	12	0.207 ± 0.03	3.04 ± 0.4	0.039 ± 0.005	0.573 ± 0.07
Col IV	22	0.072 ± 0.01	0.874 ± 0.1	0.027 ± 0.003	0.329 ± 0.04
CNF	46	0.16 ± 0.03	1.64 ± 0.3	0.055 ± 0.006	0.567 ± 0.06
LN-521	27	0.379 ± 0.04	3.76 ± 0.4	0.10 ± 0.02	0.992 ± 0.16

Figure 8. *The interactions between human collagen I (Col I), collagen IV (Col I), laminin 521 (LN-521), or cellulose nanofibrils (CNF) with human hepatocarcinoma cell line HepG2 or human pluripotent stem cell line WA07. Representative retraction force curves for HepG2 (a) and WA07 (b) and adhesion energy (c) were recorded when the materials and cells were in contact 30s before retraction. Error bars are standard errors of mean and significant differences of $p \leq 0.05$ are marked with *. Both un-normalized values and values normalized by the probe radius R are shown for HepG2 (d) and WA07 cells (e) at 30 s contact time. Modified from Publication I.*

Because cells can actively control the distribution and conformation of the integrins, and thus affect cell – biomaterial interactions, we continued the studies by probing the interactions in more detail. These spectroscopy studies were performed using only LN-521 and CNF as biomaterials, as they had shown distinct affinities for cells in terms of adhesion energy and force curve profile with both the studied cell types (Publications I and II).

Focal adhesion and integrin localization in hPSCs have shown to be unique and related to the pluripotency of these cells (Närvä et al. 2017). The results from Närvä et al.

(2017) have suggested that the cell colony edges may predominately mediate hPSC-ECM interactions. This unique localization of integrins allows us to probe both specific and nonspecific interactions of integrin substrate laminin with cells. To be able to reduce the probe-cell contact area and, thus, to better record the details in the force curves, we used a newly established small and well-defined tip with contact radius of 65 nm coated with LN-521 for these studies (Publication II).



d **WA07 - LN-521 - 10s Contact**

	Number of force curves analyzed	Adhesion energy (fJ)	SEM (fJ)	Maximum detachment force (nN)	SEM (nN)
Uncoated	10	0.013	0.006	0.050	0.009
Central areas	11	0.014	0.006	0.052	0.009
Peripheral areas	33	0.047	0.011	0.081	0.009

Figure 9. *The effect of integrin localization on human pluripotent stem cell WA07 interactions with human recombinant laminin-521. Representative force curves after 1, 10, and 30s contact times recorded in peripheral (a) and central areas (b) of the WA07 cell colony. Adhesion energy (c) were recorded when the materials and cells were in contact 30s before retraction. Adhesion energy and maximum detachment force values presented in detail after 10 s contact time (d). Error bars are standard errors of mean and significant differences of $p \leq 0.05$ are marked with *. Values were normalized by the probe radius R. Modified from Publication II where sample amounts and locations are presented in more detail.*

We observed a significant difference in the adhesion between LN-521 and WA07 cells depending on the measurement location in the cell colonies (Figure 9, Publication II). The adhesion of LN-521 to integrin-enriched, peripheral colony areas was remarkably stronger than the adhesion to integrin-deficient central colony areas after contact times ≥ 10 s. Thus, at 30 s contact times the adhesion energy of LN-521 to integrin-enriched areas was approximately seven times larger (0.53 ± 0.19 fJ compared to 0.077 ± 0.026 fJ) and the maximum detachment force four times higher (0.26 ± 0.09 nN compared to 0.065 ± 0.010 nN) than to integrin-deficient areas.

The specificity of the adhesion could be interpreted by comparing the results to uncoated silicon probes used as reference, that have nonspecific interactions with cells. The uncoated reference probes showed similar adhesion as LN-521 coated probes to the middle part of the cell colonies (no significant difference, $p > 0.05$) (Figure 9d), indicating that laminin – WA07 interactions on central areas of the cell colony were integrin-independent and nonspecific. This conclusion is further supported by the fact that the observed maximum detachment forces for uncoated probes and laminin-coated probes interacting with the central areas of the WA07 cell

colonies were mostly below the strength of a single integrin – ECM bond, which typically ranges between 50 and 100 pN in living cells (Weisel et al. 2003). This value depends on the type of molecules interacting and on the loading rate applied. In contrast, the adhesion of LN-521-coated probes on peripheral areas of WA07 cell colonies was significantly stronger than for uncoated probes with maximum detachment forces equivalent to 1-5 integrin – ECM bonds indicating specific, integrin-mediated interactions. In addition, differences in the force curve profiles were observed depending on the probed cell colony area. The jumps shown in the force curve profiles after 30 s in contact in Figure 9a were only observed in situations where we expected activated integrins to participate in the interactions. These jumps are characteristic of specific ligand – receptor binding when the cell receptor is attached to the cytoskeleton, as is the case for activated integrins. In contrast, only tethers were observed with uncoated probes or LN-521 coated probes in the middle of cell colonies, suggesting that these were typical for nonspecific interactions.

The effect of integrin activation on cell – biomaterial interactions was further studied by comparing forces obtained with living and dead cells in the presence and absence of divalent cations Ca^{2+} and Mg^{2+} in the buffer (Publication II). These ions have shown to change the activity of integrins as discussed in Section 2.3.1 and are both present in cell culture media in similar concentrations as tested here. Since both ions are present in *in vitro* cell cultures, and the concentrations and the presence of both ions together have an impact on integrin activation, we considered it to be more relevant to study the combined effect of Ca^{2+} and Mg^{2+} on cell-biomaterial interactions and, consequently, did not test the role of these ions separately. Dead cells for interaction studies were obtained by fixation with 4% PFA.

Dead cells are lacking all the integrin inside-out activating signals. In our studies, we showed that cell viability has a significant impact on the interactions of cells with LN-521 (Publication II). This effect is demonstrated by the vast difference in the magnitude of adhesive forces between LN-521 and living or dead HepG2 and WA07 cells. It can be concluded that the active control of integrin activation by living cells has a high impact on integrin-mediated cell – biomaterial interactions. Also, the presence of Mg^{2+} and Ca^{2+} ions resulted in a considerably higher adhesion between LN-521 and living HepG2 cells, showing that the activation of integrin by these cations fosters specific cell-LN-521 interactions. It is important to note that while Mg^{2+} promotes the activation of integrin, Ca^{2+} can favor or inhibit integrin activation depending on the Ca^{2+} concentration. When combining these ions in the concentrations typical for *in vitro* cell models, the overall result is an increase in integrin activity, as anticipated based on their combined use in cell culture media.

On the other hand, the adhesion between dead cells and LN-521 and CNF was slightly stronger in the absence than in the presence of Mg^{2+} and Ca^{2+} (Publication II). In principle no effect of divalent cations on dead cell – biomaterial interactions would be

expected considering that there is no activation of integrins in dead cells. However, divalent cations can affect nonspecific interactions. The adsorption of divalent cations on surfaces can provoke repulsive hydration forces between strongly hydrophilic surfaces (Pashley and Israelachvili 1984), preventing the surfaces from coming into close contact where the attractive van der Waals forces would be dominant. Divalent cations could also hinder electrostatic attractions between oppositely charged groups on cell and biomaterial surfaces.

Notably, neither the cell viability, nor the presence of divalent cations had an effect on the adhesion between cells and CNF (Publication II). These results imply that CNF has only nonspecific interactions with cells and, therefore, the ability of the material to support cell spheroid formation could rely on simply providing physical support for cells in 3D cultures where the cell-cell interactions might be greater compared to cell-material interactions. This difference between the magnitudes of cell-cell and cell-biomaterial interactions could be confirmed with further AFM spectroscopy studies.

5.2 Laminin-511 and laminin-521-based matrices support hepatic specification of definitive endoderm cells

It has been shown previously that the human liver progenitor HepaRG-ACM supports definitive endoderm (DE) cell attachment and hepatic differentiation (Kanninen et al. 2016). To obtain a chemically well-defined matrix, we characterized the critical proteins of the ACM by conventional RT-PCR and IF (Publication III). The results suggest that HepaRG ACM consists of FN, LN-511, and LN-521. In addition, Col IV α2 and α5 chains were detected, but these chains do not exist together as a combination in any known heterodimers. We created cell culture matrices using these proteins and all their possible combinations, totaling altogether seven different matrices.

Flow cytometry and IF showed the pluripotency of the used hPSCs (Publication III). After the DE differentiation step, cells formed confluent monolayers with high expression of DE markers HNF3B and CXCR4. These DE cells were then transferred to the studied ECM protein matrices and differentiated towards hepatic cells in three steps. The differentiation efficiency of the matrices at each differentiation step was identified by examining cell morphology and detecting critical hepatic progenitor and hepatic marker expression shown in Publication III.

We were not able to identify differences between any hybrid matrices made from the combinations of two or three proteins with H9-GFP cell line and thus we continued the studies with iPS(IMR90)-4 and WA07 cell lines and included single protein matrices (Publication III). The differentiation efficiency was higher with iPS(IMR90)-4 cells compared to WA07 cells. The hepatic specification of these cell lines was

efficient on most of the studied matrices. We observed that FN did not cause any significant improvement in the hepatic specification or maturation, but laminins alone were able to support the hepatic specification. The use of LN-511 and LN-521 separately or together did not remarkably alter the differentiation efficiency.

The hepatic progenitor cells differentiated on laminin matrices expressed HNF4A, CK-19, and AFP (Publication III). The cells differentiated on laminin matrices exhibited typical hepatic cell morphology after 16 days of differentiation. Also, the progenitor markers AFP and CK-19 were significantly decreased and hepatic markers ALB and CYP3A4 increased. Cytochrome P450s are important enzymes in drug metabolism, and, thus, we tested the expression and inducibility of these enzymes from the differentiated cells. We detected the high expression of CYP1A2 and CYP3A4 enzymes and the dexamethasone-mediated CYP3A4 and CYP3A7 enzyme induction was also notable.

5.3 Suspension culture support hepatic specification of human pluripotent stem cell spheroids better than cellulose nanofibril gels

It is known that chemically unmodified CNF in hydrogel form supports hPSC spheroid formation and cells in these spheroids maintain their pluripotency well (Lou et al. 2014). It has also been shown that the cells can be released from the hydrogel as intact spheroids with cellulase enzyme treatment (Lou et al. 2014). We tested if this cell culture method can be used similarly in hPSC spheroid stepwise differentiation (Unpublished results IV). The iPS(IMR90)-4 and WA07 cell lines and differentiation media were the same as in 2D studies (III), so we were able to compare the differentiation efficiency of our 3D models to 2D (Unpublished results IV).

The cells differentiated in both the 3D cell culture systems exhibited typical hepatic cell morphology and expressed hepatic markers AFP, ALB and CYP3A4 (Figure 10) (Unpublished results IV). The differentiation efficiency in both systems was notably higher compared to 2D cell culture systems. The suspension culture led to better differentiation efficiency compared to CNF hydrogel and the CYP3A4 expression reached quite similar level as for primary hepatocytes. The spheroids differentiated in CNF hydrogel also showed large and dense areas without any hepatic specification. Cells differentiated in suspension culture expressed high CK-18, but also progenitor marker AFP was still expressed. With IMR90 suspension culture IF revealed still fewer mixed cell populations with no expression of CK19 nor CK18.

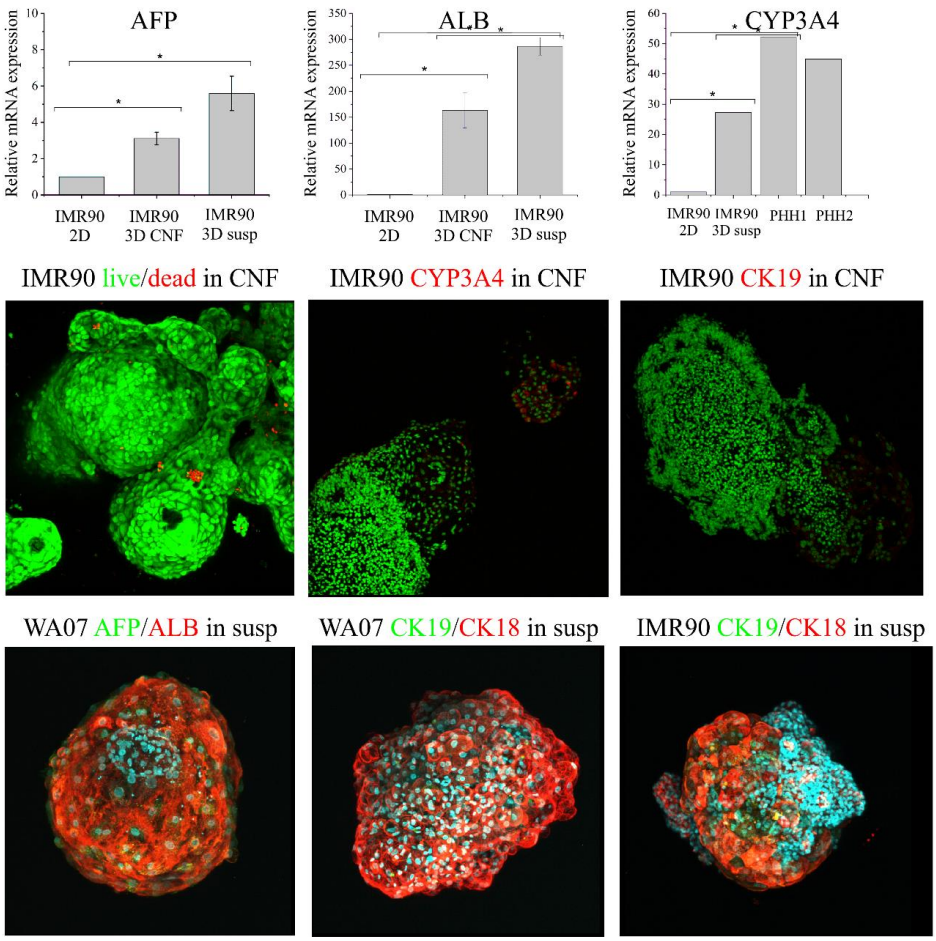


Figure 10. *iPS(IMR90)-4 (IMR90) and WA07 cell spheroids differentiated to hepatic spheroids in cellulose nanofibrils (CNF) hydrogel or in suspension culture (susp). Differentiation efficiency was compared to 2D differentiation and primary hepatocytes (PHH) and showed with relative mRNA and protein expression. AFP=alpha-fetoprotein; ALB=albumin; CK=cytokeratin. Error bars are standard deviation and significant differences of $p \leq 0.05$ are marked with *.*

6. Discussion

6.1 Colloidal probe microscopy is a useful tool to quantify cell – biomaterial interactions

AFM-based SCFS has shown to be a useful tool to study cell – biomaterial interactions that appear come when a delicate cell line, such as hPSCs, cannot be probed as single cells. The cell viability and morphology are easier to monitor during experiments with AFM-based CPM compared to AFM-based SCFS. In addition, cell – biomaterial interactions from tissue samples, as well as 3D cell aggregates or spheroids, cannot be probed with SCFS because of their large size. In this thesis, I show that AFM-based CPM offers a good, or in some cases even better, alternative to SCFS to study cell – biomaterial interactions (Publications I and II). CPM allows cell probing at a more natural state of cells since they do not need to be isolated. In addition, I show that by applying the same method with a novel tiny and well-defined cantilever tip, the interactions can be studied in greater detail compared to CPM but still allow for a more natural stage of the cells than SCFS (Publication II).

Concern has been raised about possible probe contamination in CPM suggesting that it is not a good choice for studying cell interactions (Lehenkari and Horton 1999). Tip contamination should be visible as changes in the recorded forces as the tips become contaminated. Since we did not observe any difference between the force curves recorded in the beginning or the end of an experiment, we concluded that this concern was not relevant for the systems tested here (Publication I). Also, other cell – biomaterial interaction studies have recently been performed with CPM (Chièze et al. 2019).

The sizes of the colloidal probes used were roughly similar to single cells, suggesting that the results can be qualitatively comparable to SCFS studies (Publications I and II). Unnormalized values of our studies were in the same range compared to previously reported forces (Taubenberger et al. 2014). For instance, in our studies LN-521 had a maximum detachment force of 7.04 nN with HepG2 cells and 0.99 nN with WA07 cells (Publication I) which is in line with the study of Dao et al. (2012), who reported maximum detachment forces of 0.738 ± 0.298 nN between CHO cells and laminin. Because of the strong adhesion between LN-521 and cells, we were sometimes not able to retract the cantilever from the contact with cells to obtain zero baseline and, thus, the resulted values might be slightly lower than the actual adhesion.

Since the contact area plays an important role in adhesion and to facilitate comparison between future studies using different size of probes or cells, we presented the results as normalized with the colloidal probe radius in addition to the unnormalized values

presented in supplementary data. It could be questioned whether the relatively low adhesion between HepG2 cells and Col I compared to LN-521 is due to the lack of large fibrillar morphology of produced collagen coating for the experiments and, thus, lack of functionality of this material. However, our results are similar to previously reported data using large fibrillary morphology of Col I, indicating no significant effect of Col I morphology on the adhesion of cells. For instance, the mean maximum detachment force of 1.16 ± 0.94 nN reported by us for HepG2 cells after 30 s contact time with Col I (Figure 8) is of the same order of magnitude as the >2 nN detachment forces for CHO-A2 cells after 30s contact time (Taubenberger et al. 2007) and 0.487 ± 0.315 nN for pre-osteoblastic cells after 180 s contact time (Taubenberger et al. 2010) measured by other authors using thicker Col I fibers.

Since the cells are attached to the substrate from the basolateral side and, thus, interacting with the biomaterials on the probe with their apical side, it could be questioned whether the actual integrin-biomaterial interactions with the cells are probed in these experiments. However, Schoenenberger et al. (1994) have shown that, at least in MDCK cells, integrins are located both in apical and basolateral sides. In our studies we show high and contact-time dependent adhesion with the systems expected to have specific integrin-mediated interactions, suggesting that indeed there are integrins in the apical side of the cells studied here. These results were also in line with previously reported SCFS results (Taubenberger et al. 2014) and many times higher compared to uncoated probes or tips, supporting our claims. This could still be confirmed by IF staining in future studies. In 3D, the natural stage of the cells, integrins are located on every side of the cells (Baker and Chen 2012).

3D cell culture systems resemble more closely the natural *in vivo*-like environment of the cells than 2D systems. How cell – biomaterial interactions in cell spheroids differ compared to 2D has to the best of my knowledge never been tested quantitatively. The cell – biomaterial interactions occur at the cell surface, so it could be concluded that overall the interactions are similar. On the other hand, the cell polarization and functions are different in 2D and 3D configurations. We showed that the CPM method is a useful tool to study cell – biomaterial interactions (Publication I) and, thus, also cell spheroids and tissue sections could be studied using this technique. The magnitude of cell – biomaterial interactions that support spheroid formation as well as when the adhesion is too strong to enable spheroid formation could possibly be probed in the future with this approach. These interactions could, furthermore, be compared to ACM which can also be studied with SCFS.

Coating a bigger probe in CPM instead of a small cantilever tip allowed us to use a wider range of different materials, such as CNF, which has certain restrictions in fibril flexibility. On the other hand, complex coatings, such as layered collagen membrane produced with Langmuir-Schaefer deposition, are not possible to perform on colloidal probes (Nugroho et al. 2019). In other words, the role of complex material morphology

to cell – biomaterial interactions cannot always be studied by CPM. Thus, it is important to consider, which is the optimal AFM spectroscopy method in each case.

Weak interactions are challenging to quantify and have, thus, so far been only scarcely studied. It is known that cells adhere to chemically unmodified CNF coatings poorly (Courtenay et al. 2018), and since CNF consists of glucose, CNF does not have specific binding sites to integrins. As could be expected from this fact, we detected negligible interactions between tested cells and CNF (Publication I). Further studies have shown that CNF has only integrin-independent interactions with cells, similar to those observed with uncoated probes, with no effect of the presence of divalent cations or even cell viability (Publication II). These results suggest that AFM force spectroscopy studies can be used to quantify weak and nonspecific interactions in addition to the stronger interactions more often studied.

6.2 There is a correlation between cell behavior *in vitro* and cell – biomaterial interactions measured by AFM

A clear difference in CPM results with different cell types and materials was observed; different materials gave dissimilar interactions with the same cells and vice versa (Publication I). The adhesion energy correlated best with *in vitro* cell behavior. High adhesion energy was measured between cells and biomaterials that showed high cell attachment and confluency *in vitro*. Thus, we were able to approximate the limit value of 0.23 nJ/m for cell – biomaterial interactions that resulted in cell adhesion on the material of interest and enables 2D cell culture on this material (Publication I).

The expression level of ECM macromolecule-specific integrin subtypes was predicting best the magnitude of the force between cells and biomaterials (Publication I). However, integrins are not the only receptors affecting cell – biomaterial interactions. The level of the integrin subtype expression might also vary. For instance, HepG2 cells have both collagen and laminin-specific integrins, but the adhesion to laminin was significantly higher compared to collagens. Furthermore, even though the hPSCs has shown to express $\alpha1\beta1$ and $\alpha2\beta1$ integrins for collagen, hPSC adhesion on these biomaterials have not been previously observed (Evseenko et al. 2009; Laperle et al. 2015; Miyazaki et al. 2008; Xu et al. 2001). Because of the overlapping substrate specificity of integrins and vice versa, as well as integrin crosstalk with other integrins or other cell membrane receptors and different ligand-binding mechanisms, it is not easy to know the cell behavior simply by analyzing the integrin cassette of the cells. Thus, force spectroscopy studies between cells and biomaterials are particularly useful. Previously, the quantitative role of single integrin subtypes to cell adhesion have been studied by blocking the subtypes with antibodies or peptides (Friedrichs et al. 2010; Sun et al. 2005b). The role of other cell-surface receptors, such as syndecans,

to cell – biomaterial interactions has not yet been extensively examined quantitatively. Integrin signaling has a significant role in cell survival and blocking all these receptors can have a too high effect on normal cell behavior. Thus, it is not possible to block all the integrins to study nonspecific interactions. Hence, we did not use blocking of integrins in our work but another approach as described below.

Biomaterial morphology has shown to affect cell – biomaterial interactions and further cell behavior, also in hPSC-derived cells (Sorkio et al. 2015). In our studies, Col I and Col IV that have different morphology showed similar adhesion and force profiles with the studied cells (Publication I). From these results it could be speculated that the morphology of biomaterials maybe has a lower impact on the magnitude of cell – biomaterial interactions when compared to the specific material chemistry that is responsible for the material interactions with integrins. Likewise, Abdallah et al. (2017) have shown, using surface-proteomic screening approach, that the biomaterial surface chemistry determines the interaction with cells. Biomaterial morphology additionally affects the presentation of growth factors and receptor binding sites of the material and may be the reason for altered cell response. In addition to the mechanotransduction, the specific chemical interactions are the ones that onset biological response in cells.

6.3 AFM reveals the specificity of cell – biomaterial interactions

In the studies presented in this thesis, we were able to quantitatively show the role of integrins and their activation in cell – biomaterial interactions. We used a slightly different approach to the previously reported methods as elaborated below. The role of some integrin subtypes has been studied by blocking with antibodies or peptides. Unfortunately, blocking them all at once is not possible, and antibodies may also have some nonspecific interactions with the materials. As can be seen from the work by Dao et al. (2013), blocking laminin-specific integrin with antibody does result in stronger interactions between CHO cells and laminin than cells and nonspecific reference. The unique localization of integrins concentrated at the edge areas of hPSCs colonies revealed by Närvä et al. (2017) made it possible to study integrin-independent cell – biomaterial interactions, quantify these nonspecific interactions, and compare them to specific interactions in the native state of the cells without any disturbing additives. We were able to probe both integrin-mediated interactions and nonspecific interactions of integrin substrate LN-521 and to show their quantitative role in cell – biomaterial interactions (Publication II). In addition, by probing the forces between uncoated probes and cells, it was possible to reveal the nonspecific interactions of cells and further the quantitative role of all cell adhesion receptors altogether (Publication II). A similar method to compare interaction specificity with negative controls has previously been used by Dao et al. (2013).

Interestingly, even though the cell viability is known to have a high impact on cell adhesion and, thus, the active control of the adhesion by cells is known to be important, it has never been tested previously with AFM to the best of my knowledge. Dead cells are lacking all the inside-out activation signals, and thus only nonspecific and specific non-activated interactions with biomaterials are present. Hence, we suggest that dead cells could be used to probe these types of interactions and by comparing those forces with the ones obtained with living cells to discriminate the contribution of specific activated interactions (Publication II). We can further discriminate the non-specific interactions of integrin-deficient WA07 cells from non-activated integrin interactions of dead cells with integrins.

Previously, the quantitative role of integrin conformation in cell – biomaterial interactions through divalent cations Ca^{2+} and Mg^{2+} has been studied by AFM in a small number of studies (Taubenberger et al. 2007; Trache et al. 2010). The studies were performed with animal cells, and Trache et al. have measured forces at room temperature and showen the inductive role of Mg^{2+} ions for cell adhesion to collagen I by chelating the ions. The chelating agents themselves could have some effect on the recorded forces and, thus, we preferred to use two different media with or without these cations. Trache et al. have concluded that the addition of Ca^{2+} decreases this Mg^{2+} dependent cell adhesion to the same material (Trache et al. 2010). They used ion concentrations (4 mM Mg^{2+} and 0.25 mM Ca^{2+}) that do not resemble the ones normally found in cell culture media (for instance, DMEM/F12 has 0.4 mM Mg^{2+} and 1.05 mM Ca^{2+}). The 1 x DPBS+ we used as a buffer better resembles cell culture media ion concentrations (0.49 mM Mg^{2+} and 0.9 mM Ca^{2+}), which is crucial because of the concentration dependency of the activation (Publication II). Because sensitive hPSCs start to detach and die after 10 minutes without Ca^{2+} and M^{2+} ions, it was not possible to use those cells in these studies.. The quantitative role of these divalent cations in combination and at the concentrations typically used in cell cultures was shown to induce integrin-dependent cell – biomaterial interactions (Publication II).

Low forces were detected using uncoated probes as negative controls, and the effect of cations and cell viability gave strong support to our interpretation of the interaction specificity (Publications I and II). In addition, we were able to distinguish jumps in the force-curve profiles from specific cell – biomaterial interactions, while only tethers were observed in the systems that were expected to have nonspecific interactions (Publication II). We, furthermore, demonstrated that the jumps showing specific activated interactions occurred only after longer than 10 s time in contact. It can be concluded that the specific activated integrin – biomaterial interactions develop slowly, which is in line with previous observations by Taubenberger et al. (2007). Similarly, Dao et al. (2013) have concluded that nonspecific background adhesion does not increase, or increases only minimally with contact time.

From the forces between reference samples and cells it can be easily seen that adhesion energy values correlate better with cell adhesion *in vitro* than maximum detachment forces (Publication I). Some nonspecific materials, such as APTES, show high detachment forces and peak-like force profile but lower adhesion energy (Publication I). In the literature, some materials that were given as nonspecific references, such as bovine serum albumin, have shown high adhesion on cells (Dao et al. 2013). On the other hand, even though albumin does not have interactions with cells through integrins or other adhesion molecules and does not support cell adhesion, they bind to cells with several different receptors, such as glycoproteins 18, 30, and 60 (Merlot et al. 2014) and thus the interaction between cells and albumin should not usually be considered to be nonspecific.

In dead cells only nonspecific interactions are present, and interestingly the effect of these ions was the opposite for systems using dead cells (Publication II). It has been reported that the adsorption of divalent cations on surfaces can provoke repulsive hydration forces (Pashley and Israelachvili 1984) and also hinder electrostatic attractions between oppositely charged groups on cell and biomaterial surfaces. It should be noted that cations have a role in biomaterials and their ligand binding motifs. Altogether, the divalent cations appear to reduce the nonspecific, attractive forces in dead cells (Publication II). In contrast, those ions increase the adhesion of living cells to integrin ligand like LN-521, supporting the connection of the divalent cations with the active control of integrin activation.

The combination of existing tools used in SCFS studies with CPM can provide detailed information about the interaction mechanisms that various materials with different chemistry and morphology have with cells. It is important to be able to separate nonspecific interactions from specific ones when developing new cell culture materials or hybrid cell culture scaffolds with tunable cell adhesion properties.

6.4 Tissue- and stage-specific cell – biomaterial interactions induce hPSC differentiation

As discussed in the literature part of this thesis, integrin activation is known to affect stem cell differentiation by activating intracellular signaling pathways through specific integrin subtypes. Integrin activity is also dramatically increased upon hPSC differentiation, as shown by Närvä et al. (2017). The integrin cassette and ECM content are known to change stepwise during hPSCs differentiation. The role of ECM proteins in stem cell differentiation is known, but up to this point that knowledge has not been efficiently used to improve current *in vitro* cell models.

As is already known, the stage-specific acellular matrix can be used to guide stem cell differentiation (Hoshiba et al. 2009; Hoshiba et al. 2010; Kanninen et al. 2016; Yan et al. 2015). Also, chemically well-defined ECM proteins have been shown to induce hPSC differentiation to definitive endoderm (DE) (Brafman et al. 2013). Here we show that stage-specific ECM proteins can be screened and used for inducing hPSC differentiation to hepatic lineage (Publication III). With this method, it is possible to obtain stage-specific, chemically well-defined, and xeno-free surfaces for hPSC differentiation. Chemically well-defined matrices could help to reduce batch-to-batch variability between cell cultures. Unfortunately, we were still not able to obtain mature hepatocytes with this 2D differentiation protocol (Publication III). The differentiated cells were phenotypically closer to fetal hepatocytes. This problem could be solved with more *in vivo*-like 3D cell culture methods.

6.5 The magnitude of cell – biomaterial interactions is guiding the material usage in 2D and 3D cell culture applications

It is known that in 2D cell culture applications the interactions between cells and substrate materials need to be strong enough to support cell attachment. In our studies, we show that the adhesion energy correlates well with cell adhesion in 2D for distinct cells and materials (Publication I). In 3D, the magnitude and type of interactions allowing the cells to form cell spheroids are not well known. The CNF hydrogel allows the spheroid formation of basically any cell type, including hPSCs in an undifferentiated stage. Combining this information with our results that are revealing weak and nonspecific interactions of cells with CNF gives us valuable information about the cell – biomaterial interactions needed for cell spheroid formation (Publications I and II). It seems that in weak signaling materials where cells can freely move, cell – cell contacts are more favorable and, thus, allow cell spheroid formation.

It could be concluded that the weak and nonspecific, integrin-independent signaling of CNF can help hPSCs to remain undifferentiated. Even though αvβ5 has been shown to support hPSC self-renewal (Braam et al. 2008), it could be speculated that the lack of signals is preventing stem cell differentiation, and eventually favors cells to maintain their current stage.

Matrix-based systems resemble the *in vivo*-like environment, where it is possible to have correct physical cues in addition to the required chemical and biological signals. Even though CNF has proven to be excellent material in stem cell maintenance in 3D (Lou et al. 2014), stem cell differentiation has different requirements regarding ECM signals as discussed in Section 2.4. The unique property of CNF, allowing spheroid release from the matrix with cellulase enzyme treatment without harming the cells, gave us a chance to test matrix-based stepwise hPSCs differentiation for the first time

(Unpublished results IV). In addition, CNF allows the tuning of the material stiffness by varying the CNF concentration in the hydrogel. This stiffness tuning allows the study of the role of mechanotransduction in hPSC differentiation.

Integrin activity is increased in hPSC differentiation (Närvä et al. 2017). In line with that, we have observed that tissue- and stage-specific cell – biomaterial interactions induce hPSC differentiation. The cells under differentiation do not gain the crucial biochemical signals from the CNF matrix, and thus the efficiency in CNF hydrogel is lower compared to suspension culture (Unpublished results IV). It could also be speculated that because CNF is lacking the necessary GF binding and presenting sites, some GF signaling important in hPSC differentiation may be disturbed. In addition, GF binding has shown to prolong their activity, which might be crucial in matrix-based 3D cell culture systems with restricted molecule flux.

6.6 Future prospects

To obtain effective *in vivo*-like 3D cell culture models from hPSCs for applications, we need to gain a good balance of signaling materials guiding the cell differentiation and helping the spheroid formation. For this purpose, we need new hybrid scaffolds that mimic all the crucial signals between materials and cells. Based on the results from previous studies with feeders and ACM, it can be concluded that hybrid materials have effects that cannot be seen from the single material. For instance, the role of structural proteins has been shown to be relevant even though they are not highly signaling materials. On the other hand, technical questions such as allowing robustness, as well as availability and user friendliness need to be addressed. Data from AFM force spectroscopy could be utilized in the creation of new hybrid materials by testing the adhesion and specific interactions of these materials with cells.

Even though CNF has some superior technical features and is suitable for hPSCs maintenance, it has limitations when used for hPSC differentiation. Low signaling CNF with excellent technical features could be used as a backbone in different hybrid materials combined with highly signaling ECM proteins, such as laminins or fibronectin. The stage- and cell type-specific ECM components detected with the method we introduced in this thesis could be used as these highly signaling proteins. In the case of, for instance, hepatic specifications, we could use AFM to study the correct mixing. Tuning CNF with GF binding sites is also needed to enhance the stem cell differentiation in 3D. How this kind of tuning affects cell – biomaterial interactions could be examined by AFM. We could compare the interactions of differentiated cells with hybrid materials and natural ACM with SCFS. The differentiation efficiency to hepatic lineage could be further increased under medium flow (Ramachandran et al. 2015).

7. Conclusions

Natural ECM of the cells is a highly complex array or different biomaterials and it provides various signals to cells through different cell receptors. To be able to create *in vivo*-like cell culture models, it is important to detect the key signals needed for different applications. Previously, it has been shown that in hPSC maintenance the correct integrin signaling prevents the stem cell differentiation. Here we show that weak nonspecific integrin-independent interactions of cell culture materials, such as CNF, with hPSCs can also prevent stem cell differentiation and allow cell spheroid formation. On the other hand, we show that specific integrin - biomaterial interactions are essential for guiding controlled hPSCs differentiation to somatic cell types. These are partially the underlying reasons behind the fact that, despite the many advantages of CNF, it is not a suitable material for hPSC differentiation as such.

This thesis illustrates the versatility of the AFM for cell – biomaterial cell studies and that the different types of cell – biomaterial interactions quantified with CPM have a good correlation with cell behavior *in vitro*. We indicate that the CPM method is a useful tool to study cell – biomaterial interactions and, thus, also cell spheroids and tissue sections could, in the future, be studied with AFM. The information obtained from force measurements can be utilized in creating novel hybrid materials for cell culture applications. With the correct set-up we can discriminate between nonspecific and specific interactions and target them to the certain cell-surface receptors with desired biological functions.

This thesis introduces new methods to find and characterize suitable biomaterials to boost hPSC differentiation and to develop well-defined matrices and hybrid scaffolds in 2D and, especially, 3D. We also show the means to study the specificity of cell – biomaterial interactions aiming to utilize cell – ECM interactions to induce stem cell differentiation.

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